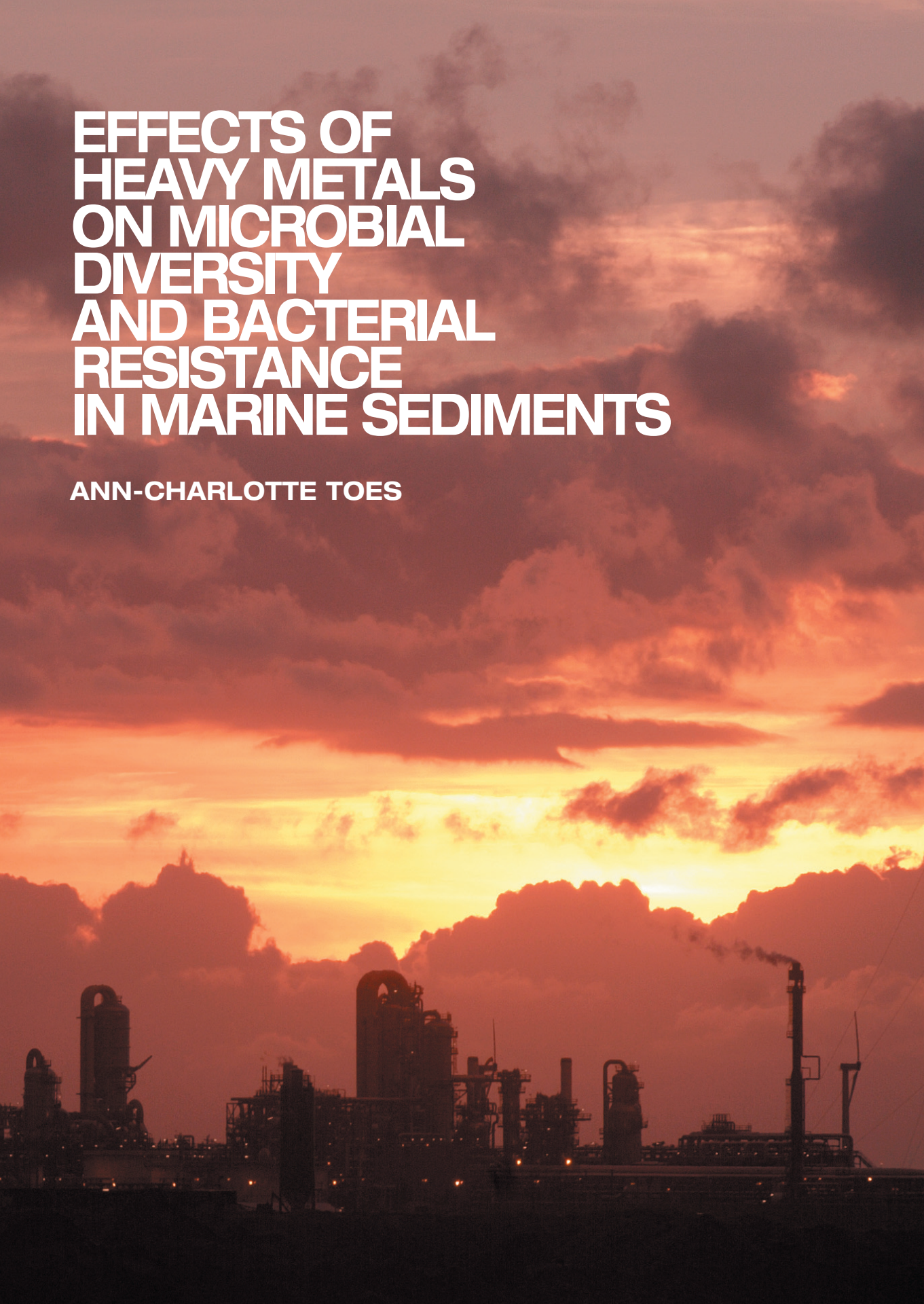
EFFECTS OF HEAVY METALS ON MICROBIAL DIVERSITY AND BACTERIAL RESISTANCE IN MARINE SEDIMENTS

ANN-CHARLOTTE TOES



Effects of heavy metals on microbial diversity and bacterial resistance in marine sediments

Proefschrift

Ter verkrijging van de graad van doctor aan de Technische Universiteit Delft, op gezag van de Rector Magnificus Prof. dr. ir. J. T. Fokkema, voorzitter van het Colleges voor Promoties, in het openbaar te verdedigen op donderdag 6 maart 2008 om 10.00 uur

door

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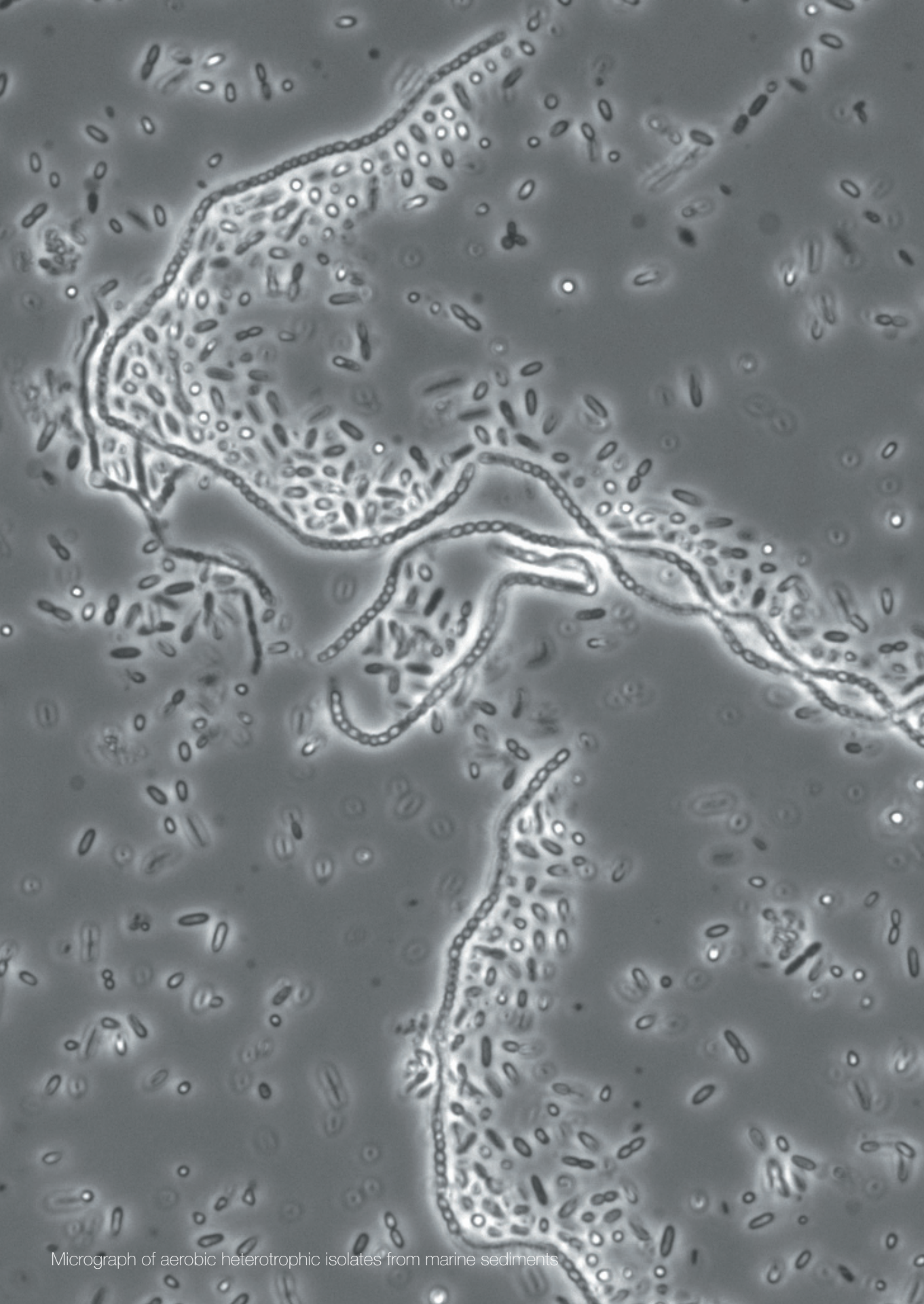


Micrograph of aerobic heterotrophic isolates from marine sediments

Summary

Different degrees of heavy metal pollution have been observed in European coastal areas, mostly attributed to industrial discharges, waste disposal streams and atmospheric deposition of exhaust gasses. Stringent environmental legislation has led to a reduction of these metal discharges in the last decades. However, considerable heavy metal pollution of sediments from harbours and marinas subsists and has been attributed to the application of antifouling paints on ship hulls. Some of these harbours have to be dredged frequently for navigational purposes, as is the case for harbours in North-Western Europe from which more than 200 million cubic meters of contaminated sludge is dredged on an annual basis. Deposition of dredged harbour sediments in relatively undisturbed ecosystems is often considered a viable option for confinement of pollutants and possible natural attenuation. This has been the practice without any knowledge of the baseline (micro)biology or the effects of heavy metal pollution of the undisturbed ecosystems.

This research started with an investigation of the effects of deposition of heavy metal–polluted sludge on microbial diversity. We developed molecular tools to study diversity and used cultivation-based approaches for isolation of bacteria. This enabled us to study relations between heavy metals and bacteria in marine sediments in a broader context. The research presented in this thesis touches three intertwined aspects of metal-microbe interactions: i) the effects of heavy metals on microbial diversity (chapters 2 and 3), ii) the potential influence of bacteria on metal bio-availability (chapter 4) and iii) microbial heavy metal resistance of marine *Shewanella* bacteria (chapters 4, 5 and 6). The first chapter of this thesis introduces the various subjects and presents a literature overview. Chapter 7 discusses the results in a broader context and presents an outlook.

Prior to determining the effects of heavy metals on marine sedimentary microbial ecosystems, control experiments were conducted to provide insight in the community structure of the undisturbed community in sediments. In chapter two, the applicability of molecular tools on sediment samples was investigated. Besides optimization of DNA extraction and subsequent polymerase chain reaction (PCR), we studied the reproducibility of a microbial community fingerprinting technique, denaturing gradient gel electrophoresis (DGGE). Four types of sediments were kept in small-scale mesocosms (~6 litres). PCR-DGGE analysis of three random surface samples showed that patterns reflecting microbial communities were generally reproducible for three of the four sediments, i.e. two fine-grained, silty sediments and one sand-silt mixture. The sandy sediment included in the study showed more spatial heterogeneity, since individual DGGE profiles showed less similarity.

Large-scale mesocosms (~72 litres), filled with sediments from a harbour basin (HB, silty) and from an intertidal flat (IF, sand) were constructed for the actual contamination experiment. Sediments in these mesocosms showed visual signs of vertical stratification, on a mm-scale below the surface, in addition to a high degree of heterogeneity. Thorough analysis of PCR-DGGE patterns of samples in the horizontal as well as the vertical direction, and phylogenetic identification of individual bands, enabled an initial characterization of the microbial communities in sediments HB and IF. Sequences related to *Shewanella marisflavi* (belonging to a family known to reduce heavy metals) were detected in several DGGE profiles obtained of both sediments, suggesting that this micro-organism is a numerically significant member of the benthic bacterial communities in HB and IF.

Chapter three describes temporal changes in metal chemistry and in microbial diversity after deposition of a 3mm layer of metal-polluted harbour sludge (HB) on a relatively undisturbed sandy sediment (IF), maintained and monitored for one year in large mesocosms. Geochemical analyses showed an initial increase in dissolved metal concentrations (i.e. iron, copper and cadmium). Profiles of concentrations of copper and cadmium in pore water, peaked at the surface or just below. These high (sub)surface concentrations were probably caused by the microbial oxidation of metal-contaminated organics at the sediment surface. After three months of incubation with bio-turbation by *Nereis diversicolor*, iron, copper and cadmium concentrations were lowered approximately 10-fold at all sediment depths, due to mixing by the polychaete worms and subsequent chemical oxidation, adsorption or precipitation.

No influence of the deposited sediment was observed in temporal DGGE profiles of bacterial 16S rRNA genes, while a minor, transient impact on the archaeal community was shown. Phylogenetic analyses of bacterial 16S rRNA clone libraries showed abundance of members of the *Flavobacteriaceae,* the α– and γ-Proteobacteria in both sediments, and revealed groups of highly similar, but not identical sequences. These clusters were most closely related to *Ruegeria atlantica, Rhodobacter sphaeroides, Vibrio splendidus* and an environmental clone within the *Flavobacteriaceae*, BrownBay 2-71 (AY133394), originating from a heavy metal-polluted Antarctic sediment . Despite the finding that some clusters of sequences were shared between the metal-impacted sandy sediment and the harbour control, the two sediments were statistically different (p=0.001) in community composition. Consequences of re-deposition of metal-polluted sediment were primarily underlined with cultivation-dependent techniques. Toxicity tests showed that after one year mesocosm incubation, the percentage of cadmium- and copper-tolerant aerobic heterotrophs was highest among isolates from the sandy sediment with a 3-mm layer of metal-polluted mud on top. Although it is unlikely that these metal-tolerant bacteria belong to numerically relevant species, as no large changes were observed in DGGE profiles, these isolates provide evidence for a prolonged modification of the indigenous bacterial community caused by transient exposure to copper and cadmium.

Toxicity of heavy metals is dependent on their bio-availability, according to the free ion activity model, which assumes that dissolved metals in the form of "free" cations are bio-available and thus toxic, whereas (in)organic metal complexes are not. In marine systems, where sulfide concentrations are relatively high due to activity of sulfate-reducing bacteria, a considerable fraction of heavy metals is expected to be immobilized as metal-sulfides under anaerobic conditions. Hypothesizing that substances as hydrous ferric oxide (HFO) and δ-MnO_2 may also represent important heavy metal reservoirs in marine systems, enrichments for Fe(III)- and Mn(IV)-reducing bacteria were inoculated with extracts of the metal-polluted harbour sludge and the undisturbed sandy sediment. By serial dilution, three *Shewanella*-like metal-reducing isolates were obtained. From the harbour sediment, strain MB4 was isolated (99% similarity to 16S rRNA gene *S. marisflavi*), when δ-MnO_2 was supplied as terminal electron acceptor. Its 16S rRNA sequence corresponded to sequences retrieved previously from clone libraries and DGGE profiles. Offering Fe(III) as electron acceptor led to the isolation of strain FB18 (98% similarity to *S. colwelliana*). From the sandy sediment, a very similar Fe(III)-reducing bacterium was isolated, strain FS8 (97% similarity to *S. collwelliana*).

Chapter four focuses on characterization of heavy metal resistance of these three isolates under different growth conditions, and their potential to mobilize heavy metals from sedimentary phases, as hydrous ferric oxides and δ-MnO_2. Results were compared to data obtained with type-strain *S. oneidensis* MR1. Under aerobic conditions the two iron-reducing isolates, FB18 and FS8, were most tolerant to cobalt and zinc, whereas strain MR1, and particularly isolate MB4, showed more resistance to copper and cadmium; toxicity tests indicated that FB18 and FS8 ceased growth at 150µM Cu, while growth of strains MB4 and MR1 under the same conditions reached 56-58±0.1% of maximal optical density, ODmax, in control cultures. Copper resistance was further investigated under anaerobic conditions with lactate as carbon source and different electron-acceptors, i.e. fumarate, HFO, δ-MnO_2. Anaerobic toxicity tests with fumarate indicated no significant change in copper tolerance in strain MB4 (66±3% ODmax at 150µM). Tolerance levels of isolates FB18 and strain MR1 were approximately similar compared to aerobic conditions, but FS8 showed increased susceptibility to copper.

Results of a bi-phasic experimental design, combining δ-MnO_2-reduction and subsequent use of fumarate, furthermore indicated that the presence of δ-MnO_2 decreased bio-availability of copper through sorption processes, thereby alleviating the toxicity of copper to strain MB4 to some extent. By offering δ-MnO_2, pre-equilibrated with copper at concentrations around maximal adsorption capacity, to strain MB4 as final electron acceptor, we showed that the onset of copper toxicity in isolate MB4 was delayed until all δ-MnO_2 had been reduced. Challenging MB4 with δ-MnO_2 incubated in copper concentrations 25-fold higher than maximal adsorption capacity, resulted in immediate toxicity during Mn(IV)-reduction. Scanning electron microscopic images showed the initial amorphous Mn(IV)-oxides and newly formed, highly crystalline, lemon-shaped, particles making up the precipitate that remained after microbial reduction. Concomitant electron dispersive x-ray spectrometry confirmed presence of copper in the initial sample, yet detected no copper in the precipitate after microbial reduction, indicating that the Mn(IV)-reducing Shewanella strain MB4 immobilized copper adsorbed to δ-MnO_2.

Copper homeostasis is tightly regulated in all living cells as a result of its necessity and toxicity in free cationic form. In Gram-negative bacteria, protein systems enabling copper transport across the cytoplasmic and outer membrane, play an important role in copper resistance. Two well studied examples of copper transporters in *E. coli* are copA, a CPx-type ATPase, and cusA, part of a multi-subunit protein complex belonging to the Resistance, Nodulation and Division protein family. A closer look at the annotated genome of *S. oneidensis* MR1 revealed the presence of *cusA*- and *copA*-like genes. Primers were designed to amplify small nucleotide fragments of the corresponding genes in strain MR1 and related *Shewanella*. Chapter five focuses on the application of these primers in Quantitative-PCR, in order to monitor expression of *copA* and *cusA* in RNA extracts of MB4 and MR1 during growth with copper.

Q-PCR results of four individually treated, biological replicates of cultures of MB4 and MR1, harvested at OD600 0.2 (early exponential phase), indicated moderate reproducibility between biological replicates (2-12% deviation), not uncommon in studies of in vivo gene expression. Reproducibility between technical replicates was high (0.1-0.8%). Under aerobic growth conditions, the correlation between the presence of copper (25μM) and expression of presumed copper determinant *cusA* was significant in both MB4 and MR1 ($p=0.0006$ and $p=0.0001$, respectively). *CopA* expression in MR1 in presence of copper was not significantly different from controls under the tested conditions ($p=0.067$). Further aerobic tests pointed out that cusA expression in MB4 increased approximately 2-fold between 25 and 100μM of copper, and was also significantly induced by cadmium ($p=0.0013$). *CusA* was detectable in MR1 under all conditions tested and copy-numbers were significantly higher in cultures grown in presence of either copper or cadmium (all $p<0.0001$). When anaerobic growth conditions were applied and fumarate was offered as terminal electron acceptor, *cusA* expression in presence of 100μM of copper was lower than under aerobic conditions, but still significantly higher than controls (MB4: $p=0.038$, MR1: $p=0.0059$). Interestingly, *copA* was only significantly induced under anaerobic conditions ($p<0.001$) in MR1. This data suggests essentially different roles for the two proteins *copA* and *cusA* in the copper response in *S. oneidensis* MR1, similar to findings in more metal-resistant bacteria as *E. coli* and *C. metallidurans.*

Finally, the developed Q-PCR assay for *Shewanella*-like *copA* and *cusA* fragments, was tested on DNA extracts from various marine sediments, as described in chapter 6. Goal of this study was to investigate whether quantification of genes encoding microbial copper transporters could form a suitable strategy to assess bio-available copper in marine sediments. Initial Q-PCR assays indicated that PCR amplification efficiency of molecular standards for *copA* and *cusA* was linear across 7 orders of magnitude, with minimal detectable concentrations of 10 copies/reaction. Serial dilution of target DNA, obtained from a metal-polluted harbour sediment was subjected to Q-PCR to estimate effects of co-extracted PCR-inhibiting compounds. This resulted in linear efficiencies across a relatively broad range of input DNA (0,1-23ng DNA). Primer specificity was analyzed by cloning the PCR products, amplified from HB sediment target DNA, and creating a clone library for both *copA* and *cusA*. Subsequent analysis of the inserts of 10 individual clones showed very high sequence resemblance (average 0.02 and 0.005 number of base differences per site, for *copA* and *cusA* clone libraries, respectively).

Microbial community DNA extracted from 11 different North Sea sediments was used as target in the Q-PCR assay for analysis of *copA, cusA* and 16S rRNA genes; the strongest correlation was found between copy number *cusA* and total extracted copper ($R^2 = 0,78$), but only when the 4 sandy sediments were excluded. For *copA* no significant correlation with copper was found. Further analysis of microbial DNA extracted from sediment samples taken along a depth profile (5mm interval) in the HB sediment, revealed that the number of 16S rDNA genes decreased with depth from $1*10^{10}$ to $6*10^{9}$ per g of wet sediment. *Shewanella copA* fragments ranged from 10-4400/g of wet sediment, and the *cusA* genes from 60-660/g of sediment. Statistical analysis of the Q-PCR results indicated that *cusA* and *copA* copy numbers were not uniformly distributed with depth, nor correlated with pore water copper concentrations or total extracted copper.

Samenvatting

Vervuiling met zware metalen in Europese kustgebieden wordt voornamelijk veroorzaakt door industriële en gemeentelijke afvalstromen en afzetting van uitlaatgassen vanuit de atmosfeer. Strengere milieuwetgeving heeft in de laatste decennia geleid tot een vermindering van metaalvervuiling in kustgebieden in het algemeen. Havens vormen hierop een uitzondering. Vanwege het gebruik van metaalhoudende verf, die corrosie van de scheepsromp tegengaat en aanhechting van schelpdieren voorkomt, zijn de sedimenten van havenbassins vaak ernstig vervuild met zware metalen. Voor een aantal van deze havens is frequent baggeren essentieel voor het bevaarbaar houden van de vaarwateren. Per jaar wordt alleen al in Noordwest Europa 200 miljoen kubieke meter vervuild sediment gebaggerd. Depositie van vervuild havensediment in relatief onverstoorde ecosystemen, wordt vaak gezien als een acceptabele optie. Men neemt aan dat dit geen significant risico oplevert voor het ecosysteem, mits de metalen niet kunnen vrijkomen, maar in feite is niet veel bekend van de effecten die deze depositie heeft op het (microbiële) (eco)systeem

Dit project is begonnen met een studie naar de effecten van depositie van vervuild havenslib op microbiële diversiteit. In dit onderzoek zijn moleculaire technieken toegepast, gebaseerd op het vertalen en vergelijken van de codes in microbieel DNA, mRNA en eiwitten. Daarnaast zijn verschillende bacteriën uit vervuilde havensedimenten geïsoleerd in laboratoriumculturen, voor het bestuderen van individuele metaaltolerantie. Het onderzoek in dit proefschrift richt zich op drie aan elkaar gerelateerde aspecten van metaal-microbe interacties: i) de effecten van zware metalen op biodiversiteit (hoofdstukken 2 en 3), ii) de mogelijke invloed van bacteriën op de beschikbaarheid van metaal (hoofdstuk 4) en iii) de microbiële resistentie tegen zware metalen in *Shewanella* bacteriën (hoofdstukken 4, 5 en 6). Hoofdstuk 1 introduceert de verschillende onderwerpen en geeft een overzicht van bestaande literatuur. In hoofdstuk 7 worden de resultaten besproken en in een bredere context gezet.

Voordat enige veranderingen in biodiversiteit in marine sedimenten vastgesteld kon worden, was het noodzakelijk controle experimenten uit te voeren om het onverstoorde systeem grondig te analyseren. In hoofdstuk 2 staat de toepasbaarheid van moleculaire technieken centraal, samen met de reproduceerbaarheid va deze technieken op sedimentmonsters. Allereerst betrof dit de optimalisatie van DNA-extractie uit sedimenten en de daaropvolgende amplificatiereactie (Polymerase Chain Reaction, PCR). Het DNA is vervolgens onderworpen aan Denaturant Gradient Gel Electrophoresis (DGGE), een methode waarbij een “vingerafdruk” wordt gecreëerd van alle bacteriën in een sedimentmonster. In kleine aquaria (6 liter), voor twee derde gevuld met havensediment, is getest hoe vergelijkbaar de DGGE profielen waren van 3 willekeurige monsters. De structuur van het sediment bleek een duidelijke invloed te hebben op de heterogeniteit binnen het sediment. Drie sedimenten, die geheel of gedeeltelijk uit kleideeltjes bestonden, lieten zeer vergelijkbare DGGE profielen zien. Dit suggereerde dat de populatie samenstelling van bacteriën, archaea en cyanobacteriën homogeen was in deze systemen. De resultaten van een zanderige sediment, dat op dezelfde wijze is getest, toonden een sedimentstructuur met meer heterogeniteit.

Grootschalige aquaria (72 liter) zijn speciaal geconstrueerd voor het bestuderen van de effecten van depositie van met metalen vervuild slib (HB sediment) op een zanderig sediment (IF sediment). Door verschillende PCR-DGGE profielen van deze sedimenten te genereren, zowel in horizontale als in verticale richting, is meer inzicht verkregen in de variatie in microbiële populaties. Daarnaast heeft het uitsnijden van individuele DGGE bandjes, via DNA-analyses, de identiteit van een aantal van de meest voorkomende bacteriën onthuld. Zowel in havensediment HB als in zanderig sediment IF, werd DNA aangetroffen behorende tot de bacteriesoort *Shewanella*, hetgeen doet vermoeden dat dit type bacterie behoort tot numeriek significante populaties in beide ecosystemen. Dit is met name interessant omdat van deze groep van bacteriën bekend is dat ze zware metalen kunnen reduceren.

In hoofdstuk 3 worden de veranderingen in geochemie en biodiversiteit beschreven, ten gevolge van depositie van een 3mm dikke laag vervuild slib (HB) op een kolom van 10cm zanderig sediment (IFD), gemeten gedurende één jaar incubatie in de grootschalige aquaria. Metaalanalyses lieten zien dat in eerste instantie sprake was van een toename in de opgeloste metaalconcentraties, met name de concentraties van ijzer, koper en cadmium in poriewater. Concentratieprofielen van koper en cadmium uitgezet tegen de diepte van het sediment, vertoonden maximale waarden aan de oppervlakte van het sediment of juist daaronder. Dit duidt waarschijnlijk op metaalmobilisatie door microbiële oxidatie van organisch materiaal dat zware metalen bevat.

Aangezien normaliter in zanderige sedimenten wormen voorkomen die tunnels graven, is een groot aantal wormen (*Nereis diversicolor*) "uitgezet" in de aquaria met sedimenten in het laboratorium. Na drie maanden van actieve bio-turbatie (menging van gronddeeltjes) door de wormen, waren de concentraties van koper, cadmium en ijzer gezakt tot ongeveer 10% van de vorige waarde. Naar verwachting speelt niet alleen het fysiek mengen van de sedimentdeeltjes speelt hierbij een rol, maar vooral ook het feit dat er verschillende chemische gradiënten heersen tussen de zone waarin zuurstof kan doordringen en de anaërobe zone daaronder (<1cm diepte). Wanneer metalen in deze diepere zone worden gebracht is het zeer waarschijnlijk dat ze geïmmobiliseerd worden, door chemische oxidatie, precipitatie (b.v. als sulfide of hydroxide) of adsorptie.

Met PCR-DGGE zijn geen tijdgerelateerde veranderingen als gevolg van sediment depositie vastgesteld in de populatie van bacteriën, maar is wel een tijdelijk effect waargenomen op de populatie van archaea. Phylogenetische analyse van grote collecties 16S rRNA klonen toonde aan dat de bacteriële populaties in beide sedimenten gedomineerd werden door leden van de *Flavobacteriaceae*, gevolgd door sequenties behorende tot de α- en γ-subklasses van de Proteobacteria. Het statistisch vergelijken van het DNA van de twee sedimenten toonde aan dat, hoewel de populaties significant verschilden (p=0.001), een aantal 16SrRNA klonen bijna identiek was, waaronder een sequentie gerelateerd aan een bacterie, die tot dan toe alleen was gedetecteerd in een met zware metalen vervuild sediment op Antarctica (Brown Bay 2-71, AY133394).

Het kweken van bacteriën uit de sedimenten, maakte het mogelijk op grote schaal metaalresistentie te testen. De resultaten van deze experimenten toonden aan dat er een significante toename was in cadmium- en koperresistente bacteriën in het zanderige sediment, na depositie van vervuild haven slib. Het percentage koperresistente bacteriën in dit sediment (IFD) was zelfs hoger dan dat in het originele havensediment (HB). Aangezien de DGGE data niet duidden op grote veranderingen in populatie, is het onwaarschijnlijk dat deze metaalresistente bacteriën numeriek heel belangrijk zijn in het onderzochte sediment. Toch levert dit resultaat indirect bewijs voor een langdurige verandering van de bacteriële samenstelling, die zelfs een jaar na depositie nog aantoonbaar is.

De giftigheid van een zwaar metaal wordt in de eerste plaats bepaald door de beschikbaarheid van het metaal. Wanneer metaal voorkomt in opgeloste vorm, als "vrij" kation, kan het de celmembraan passeren. Bacteriën kunnen daarvan hinder ondervinden. Als een metaal daarentegen deel uitmaakt van een (in)organische verbinding, is het metaal niet beschikbaar en dus niet giftig (free ion activity model). In anaërobe marine sedimenten, waarin sulfide concentraties hoog zijn vanwege sulfaatreducerende bacteriën, wordt verwacht dat zware metalen grotendeels geïmmobiliseerd zijn in de vorm van metaalsulfiden. Anderzijds, zouden oxides van ijzer en mangaan, die in het marine milieu veel voorkomen, ook een belangrijk reservoir kunnen vormen voor zware metalen. Teneinde deze laatste theorie te testen, zijn kweken gestart met materiaal van beide sedimenten (HB en IF) en condities selectief voor bacteriële Mn(IV) en Fe(III) reduceerders. Door seriële verdunning zijn uiteindelijk 3 isolaten verkregen. Bacterie MB4

is afkomstig uit havensediment HB en groeit door δ-MnO_2 als terminale electronen-acceptor te gebruiken. De code van het 16S rRNA gen is voor 99% gelijk aan die van *Shewanella marisflavi* en lijkt sterk op de eerder gedetecteerde DGGE sequenties. Het aanbieden van gehydrateerde ijzer oxides (HFO) als enige electronen-acceptor resulteerde in isolatie van bacterie FB18 (98% gelijk aan *S. colwelliana*). Kweekexperimenten met het zanderige IF sediment en met hetzelfde ijzersubstraat leverden bacterie FS8 op (97% gelijk aan *S. colwelliana*).

In hoofdstuk 4 ligt de focus op karakterisering van metaalresistentie van deze 3 bacteriën, en hun potentie om koper, geadsorbeerd aan HFO of δ-MnO_2, in oplossing te brengen. Resultaten werden vergeleken met data verkregen met de verwante en grondig bestudeerde bacterie *Shewanella oneidensis* MR1. Onder aërobe condities waren FB18 en FS8 het best bestand tegen kobalt en zink, terwijl MR1 en vooral MB4 een hogere resistentie tegen koper en cadmium vertoonden. Bij een concentratie van 150mM koper, werd geen groei van FB18 en FS8 geobserveerd, maar MR1 en MB4 behaalden nog steeds 56-58±0.1% van de optische dichtheid van controle cultures. Koperresistentie werd meer in detail onderzocht onder anaërobe condities, met lactaat als koolstofbron en verschillende electronen-acceptoren: fumaraat, HFO en δ-MnO_2. De testen met fumaraat wezen uit dat koperresistentie in MB4 onveranderd was (66±3% van de maximale optische dichtheid) vergeleken met de situatie waarbij zuurstof wel aanwezig was. Bij de andere drie bacteriën werd een ietwat verhoogde gevoeligheid voor koper waargenomen.

Om te testen of δ-MnO_2 invloed uitoefent op de beschikbaarheid van koper, is een 2-fase experiment ontworpen, waarbij, na δ-MnO_2 reductie, groei verder gestimuleerd werd door het gebruik van fumaraat. Door koper van tevoren aan δ-MnO_2 te laten adsorberen in verschillende Mn:Cu verhoudingen, konden de grenzen van het systeem worden verkend. Kleine hoeveelheden koper hadden pas effect in de fumaraat fase, wat suggereerde dat het koper pas vrijkwam na volledige reductie van het mangaanoxide. Het toevoegen van grotere hoeveelheden koper, dat wil zeggen hoger dan de maximale adsorptie capaciteit van δ-MnO_2, had een acuut effect op de 1e fase van het experiment. Door monsters mangaanoxide voor en na het experiment te analyseren met een Scanning Electron Microscope, werd de transformatie van het initiële amorfe oxide zichtbaar gemaakt. Na microbiële reductie bleef een fijn residu over. Onder de microscoop bleek dit een hoog kristallijne materie bestaande uit citroen-vormige deeltjes. Element analyse bevestigde de aanwezigheid van koper in het eerste monster, maar kon geen koper detecteren na reductie, indicatief voor mobilisatie door *Shewanella* MB4.

Omdat alle cellen een kleine hoeveelheid koper nodig hebben om enzymen te laten functioneren, maar koper giftig is bij hoge hoeveelheden, wordt het passeren van koperionen over het bacteriële membraan streng gereguleerd. In Gram-negatieve bacteriën, spelen met name eiwitsystemen die koper exporteren een belangrijke rol in koperresistentie. Twee goed beschreven voorbeelden in *E. coli* zijn copA, een ATPase, en cusA, onderdeel van een groter eiwitsysteem. Annotatie van het genoom van Shewanella oneidensis MR1 wees uit dat dit type bacterie ook *copA*- en *cusA*-achtige genen bezit. Oligonucleotiden ("primers"), ontworpen om kleine fragmenten van desbetreffende genen te analyseren met Quantitative-PCR, zijn toegepast om te meten hoeveel kopieën van cusA en copA in *Shewanella* aanwezig zijn. Het gebruik van deze primers maakte het uiteindelijk mogelijk om de *cusA* en *copA* genexpressie te volgen in mRNA van bacteriën MB4 en MR1 gedurende groei met en zonder koper. Deze resultaten worden besproken in hoofdstuk 5.

De eerste resultaten met Q-PCR gaven aan dat 4 individueel behandelde cultures, waarvan de cellen geoogst waren tijdens de vroege exponentiële fase, redelijk reproduceerbare resultaten opleverden (2-12% afwijking), niet ongewoon in in-vivo gen expressie studies. De reproduceerbaarheid van de Q-PCR techniek op zich was daarentegen bijzonder goed (0.1-0.8%). De expressie van *cusA* was gecorreleerd aan de aanwezigheid van koper in het cultuur medium (25μM) in zowel MB4 als MR1 (respectievelijk p=0.0006 en p=0.0001). *CopA* expressie in MR1 was in deze situatie niet significant anders (p=0.067) dan onder controle omstandigheden. Verdere tests wezen uit dat het aantal kopieën *cusA* in mRNA van MB4 twee keer zo hoog was in 100μM koper, en dat translatie van het gen ook gestimuleerd werd door toevoeging van cadmium (p=0.0013). In afwezigheid van zuurstof, met fumaraat als electronen-acceptor, bleek cusA expressie in de bacteriën lager dan onder aërobe condities, maar nog steeds significant hoger dan onder controle omstandigheden (MB4: p=0.038, MR1: p=0.0059). Opmerkelijk was de observatie dat copA alleen significant tot expressie wordt gebracht in MR1 in afwezigheid van zuurstof (p<0.001). Eerdere studies met metaalresistente bacteriën als *E. coli* en *C. metallidurans*, wezen uit dat de eiwitten

copA and cusA essentieel verschillende rollen vervullen in de cellulaire reactie op de aanwezigheid van koper. De hier besproken resultaten laten zien dat dergelijke beschermingsmechanismen ook bijdragen aan kopertolerantie in *S. oneidensis* MR1.

Tot slot, is het ontwikkelde Q-PCR protocol voor *Shewanella*-achtige *copA* en *cusA* fragmenten getest op een collectie van 11 verschillende Noordzee sedimenten, zoals beschreven in hoofdstuk 6. Het doel van dit onderzoek was te evalueren of kwantificering van het aantal bacteriële koper exporter genen in sedimenten, een mogelijke strategie zou kunnen zijn voor het bepalen van de hoeveelheid bio-beschikbaar koper. Initiële Q-PCR tests wezen uit dat minimaal 10 kopieën per reactie nodig waren voor een signaal van het sedimentmonster en dat de efficiëntie van de amplificatie reactie lineair was tussen 10 en 10^7 moleculen input DNA. Verdunning van DNA, verkregen uit met metalen vervuild sediment, werd gebruikt om de effecten van PCR-inhiberende substanties in te schatten die per ongeluk mee geëxtraheerd zijn. Hierdoor werd duidelijk dat lineaire efficiëntie van de amplificatie reactie mogelijk was bij input DNA concentraties tussen de 0.1 en 23ng. De specificiteit van de ontworpen primers is geëvalueerd door het PCR product, verkregen uit sediment DNA *cusA*/*copA* amplificatie, te kloneren in *E. coli*. Het vervolgens vergelijken van de DNA code in 10 individuele klonen, toonde aan dat de primers zeer specifiek waren voor *Shewanella*-achtige bacteriën (gemiddeld 0.02 tot 0.005 aantal base verschillen per locatie, voor *copA* en *cusA*, respectievelijk).

Verschillende Noordzee sedimenten, variërend tussen sedimenten uit een jachthaven, een industriële haven en een natuurgebied, zijn onderworpen aan metaalanalyse en de Q-PCR test voor *copA*, *cusA* en 16S rRNA genen. De collectie van deze 11 sedimenten bevatte zowel exemplaren van zanderige sedimenten als monsters met een hoog kleigehalte. De zoutconcentraties liepen uiteen van marien tot brak. Een sterke correlatie werd aangetoond tussen het aantal *cusA* kopieën en totaal geëxtraheerd koper (R^2=0.78), maar was alleen geldig als de zanderige sedimenten buiten beschouwing werden gelaten. Verdere analyse van microbieel DNA, verkregen uit sedimentmonsters op verschillende diepten (5mm interval) van het HB sediment, gaf aan dat het aantal 16S rDNA kopieën afnam van 10^{10} per gram nat sediment in de bovenste laag, tot $6*10^9$ op 4cm diepte. Het aantal *Shewanella copA* fragmenten varieerde van 10 tot 4400 per gram nat sediment. Voor *cusA* lag deze waarde tussen de 60 en 660 kopieën per gram sediment. Statistische analyse van de Q-PCR resultaten wees uit dat het aantal *cusA* en *copA* kopieën niet uniform verdeeld was over de diepte van het sediment, maar kon geen verband aantonen tussen het aantal *cusA* of *copA* genen en de concentraties opgelost koper.

Collected metal objects on board of dredging ship

1 Introduction

Manuscript in preparation

Metals

Metallic elements

The majority of elements in the periodic system are classified as metals, with the exception of 18 (i.e. H, C, N, O, P, S, Se, the halogens and the inert gasses). Metals distinguish themselves from other elements by several physical attributes, such as the ability to conduct heat and electricity, malleability (can be shaped into sheets and wires) and lustre (shininess). Also, most metals are solid at room temperature. Specific chemical traits include the fact that metals carry one to three electrons in their outer shell, whereas non-metals have four to eight. Concomitantly, metals "lose" their valence electrons easily, contrasting with non-metallic elements, which prefer to gain or share electrons. Metals form cations in aqueous solution, have a low electro-negativity and are good reducing agents.

The metals can be further divided into several major groups, three of which are mentioned in more detail in this thesis. Groups three to twelve in the periodic system (or d-block, in IUPAC style), comprise the largest subgroup of metals, commonly known as transition metals, as discussed in the next paragraph. The metalloids are a group of elements that share some characteristics with the metals as well as with non-metallic elements. The metalloids are found on a diagonal line across the p-block and include elements as silicon, arsenate and antimony. Metalloids form amphoteric oxides (can react either as a base or as an acid) and often behave as semiconductor. The remaining group of metals is located in a triangle between the transition metals and the metalloids, called poor metals or post-transition metals, including aluminium, tin and lead. These metals are more electronegative than the transition metals; in comparison, melting and boiling points are lower and poor metals are softer.

Transition metals

Transition metals are officially defined as metals with an electronic configuration with full outer orbitals and the second outermost orbital incompletely filled, therewith excluding Zn, Cd and Hg. Elements with atomic numbers 1 through 20 have only electrons in s and p orbitals, with no filled d orbitals in their ground states. In the fourth period (or row), elements with atomic numbers 21 to 29 (Sc to Cu) have a partially filled d orbital. The outer ns orbitals in the d-block elements are of lower energy than the (n-1)d orbitals. As atoms occur in their lowest energy state, transition metals tend to have their s orbitals filled with electrons. Thus, these elements all have two electrons in their outer s orbital, with the exception of copper ($[Ar]4s^{1}3d^{10}$) and chromium ($[Ar]4s^{1}3d^{5}$).

Many properties of the transition metals are due to the ability of these d orbital electrons to delocalise within the metal lattice. In metallic substances, the more electrons shared between nuclei, the stronger the metal. Hence, transition elements tend to have high tensile strength, density, melting and boiling points. Transition metals are more electronegative than other metals and easily form covalent bounds. In addition, other metals form salts (such as $MgCl_2$), whereas transition metals form complexes (such as $FeCl_4^{2-}$ions with excess negative ions). When mixed with neutral molecules (water or ammonia), transition metals form stable compounds, while salts of other metals (e.g. NaCl) dissolve. Another characteristic of transition metals is the ability to form coordination complexes, compounds in which molecules or ions

form coordinate bonds to a central metal atom or ion; coordination complexes may contain positive ions, negative ions or neutral molecules. Finally, transition metals can have different oxidation states, a feature that makes them very useful as catalysts.

Heavy metals

Heavy metals are simply heavier than other metals, since they are roughly defined as all metals with a density above 5 g/cm^3 (Nies, 1999). This definition broadly covers most of the transition metals and a few others, i.e. from V to As (row 4), from Zr to Sb (row 5), the Actinides and the Lanthanides (rows 6 and 7). Some authors use a more narrow definition, only comprising the elements between Cu and Bi with specific gravities greater than 4, excluding essential trace elements as Fe, Co and Ni. When considering eco-toxicological effects, usually only a few heavy metals are under investigation, since a metal needs to be "bio-available" in order to be toxic for a living cell. Availability of a metal is largely determined by its solubility and it's natural abundance on Earth. Therefore, the collection of ecologically important heavy metals can be minimised to 17 elements: Fe, Mn, Mo, Zn, Ni, Cu, V, Co, W, Cr, As, Ag, Sb, Cd, Hg, Pb and U. These metals are present in the environment in concentrations high enough to have some kind of effect on living cells, either beneficial or toxic. This thesis primarily focuses on microbial interactions with selected heavy metals as iron, manganese, copper, cadmium, cobalt and zinc.

Essential metals and trace metals

Potassium, the major metal cation in all living cells, together with sodium, magnesium and calcium are essential metals. They are involved in several cellular processes, as establishment and maintenance of membrane potential, and are of evident structural importance for membranes and skeletons. Except for sodium, which rarely accumulates inside bacterial cells, average cell concentration lies in the range of 250mM (K^+), 10-20mM (Mg^{2+}) to 0.1μM (Ca^{+2}) (Barton et al, 2007). The trace metals, or micronutrients, are cellular constituents with concentrations 10-1000 fold lower. Most important among these trace metals is iron, followed in order of intracellular abundance by manganese and zinc. Copper, molybdenum, cobalt and nickel are detected in smaller quantities and may vary considerably among different Bacteria and Archaea. All trace metals are (heavy) transition metals, indispensable for function of numerous types of proteins. The evolutionary origin of these types of metal-clusters in biocatalysis is discussed in a later section, but it should be emphasised that in fact metalloproteins still make up a third to a half of all known proteins (Butler, 1998).

In order to synthesise metallobiomolecules, micro-organisms need to collect trace metals from the extracellular environment. In terrestrial ecosystems, trace metals are relatively abundant. Contrastingly, analysis of a litre of seawater reveals a relatively poor environment considering nutrients, essential metals and trace metals. Four classes of heavy metals can be differentiated based on their concentrations in seawater: frequent elements (1μM-100nM; Fe, Mo and Zn), less frequent elements (100nM-10nM; Ni, Cu, As, V, Mn, Sn, U), rare elements (10nM-1nM; Co, Ce, Ag, Sb) and very rare elements (less then 1nM; Cd, Cr, W, Ga, Zr, Hg, Pb). As a general rule of thumb, biological systems require trace metals in more or less the same concentrations as can be found in a litre of seawater (Nies, 1999). Accordingly, iron, the most abundant metal, is used in significant amounts by organisms, whereas a very rare metal as Cr or Hg would under normal circumstances not play any role in a bacterial cell.

The surface waters of open oceans are often depleted in trace metals, reflecting the uptake of these elements by actively growing marine phytoplankton, whereas at lower depths, concentrations increase due to lower cell numbers (Morel & Price, 2003). Iron-limitation may be the growth limiting factor in some oceanic waters, since amendment of iron (1-2nM) triggered an increase in primary production on separate occasions and locations (Butler, 1998). Recent genomic analyses among 122 Bacteria and 19 Archaea suggested that the number of genes encoding cystein, the main metal binding amino-acid residue, is similar in different types of micro-organisms although some variation was encountered, depending on growth conditions as nutrient supply, temperature and oxygen level (Bragg et al, 2006). Based on this observation, Barton and co-authors hypothesised that similar sulphur contents implied comparable iron content and hence, when the concept metallome was coined, defined as all metallic elements within

a cell, mean values for all trace metals were normalised to iron as shown in equations 1 (Bacteria and Archaea) and 2 (Eukaryotic Algae) (Barton et al., 2007).

$$Fe_1Mn_{0.3}Zn_{0.26}Cu_{0.03}Co_{0.03}Mo_{0.03} \quad \text{(Eqn 1)}$$

$$Fe_1Mn_{0.53}Zn_{0.08}Cu_{0.05}Co_{0.03}Mo_{0.003} \quad \text{(Eqn 2)}$$

The stoichiometry of trace metals in biomass depends on specific metabolic requirements and growth conditions. For instance, photosynthesis requires manganese cations which are therefore detected more frequently in Algae and cyanobacteria, than in bacteria.

Metals in the natural environment

Early Earth and geomicrobiology

When life arose on Earth, approximately four billion years ago, environmental conditions on this planet probably carried no resemblance to today's climate. According to some, the first "cell" was a (thermophilic) chemolithoautotroph, living on CO and an inorganic energy source. Chemistry of the ancient sea where it might have originated was controlled by volcanism and hydrothermal vents; Presumably, the water was warm and anaerobic, containing primarily reduced iron, nickel, sulphide, CO_2, ammonia, W(IV) and other heavy metals (Wächtershäuser, 2006; Nisbet & Sleep, 2001). The first organism might have made use of a metabolism involving energy-yielding pyrite (FeS_2) formation from iron sulphide (FeS) with a DG^0 of -171kJ/mole at room temperature. The catalytic activity of derived Fe-S (and Ni-S) clusters proved so valuable, that organisms still employ these metal-coordination complexes in proteins today. These clusters provide enzymes with the abilities to i) transfer electrons (oxidases and reductases), ii) bind oxyanionic and nitrogenous metabolites (nitrogenases and hydrogenases) and iii) supply a crystallisation surface for polymerisation (Imlay, 2006).

The emergence of oxygenic photosynthesis involved a 4Mn-O (III/IV) cluster as catalyst. This may be explained by the fact that manganese is one of the few transition metals that prefers oxygen as a ligand above sulphide, a feature that would diminish the concentration of all other metals considerably by precipitation (except for Fe and Ni). Geochemical analysis of banded iron formations show that oxygenic photosynthesis may have occurred locally as early as 3.8 billion years ago, but the abundance of reducing equivalents (Fe(II), HS^-, MoS_2, Cu(I)) would have limited the accumulation of oxygen in the atmosphere considerably. Notably, banded iron formations could have been shaped by (a combination of) several chemical and biological reactions, as abiotic photo-oxidation, anaerobic photosynthesis, microbial iron reduction and nitrate-dependent iron-reduction (Weber et al, 2006). One important factor in the seemingly delayed evolution of the aerobic way of life could lie in the harmful side-products derived through Fenton chemistry; reactive oxygen intermediates as hydrogen peroxide, superoxide and hydroxyl radicals, are produced during electron transfer and may react with a ferrous iron catalyst (equation 3). Other metals as copper, cobalt and manganese can also catalyse this process when present in a non-bound state in the cell. More radical species can be formed in subsequent reactions, whereby iron may be reduced again and thus the catalyst recycled (equations 4 and 5). These reactive intermediates damage proteins, DNA, RNA, lipids and sugars, and therefore enzymatic systems had to be developed to prevent posing threats. As a result, catalases, peroxidases and superoxide dismutases evolved, containing Fe, as well as Mn, Cu and Zn.

$$H_2O_2 + Fe^{2+} \text{ --> } Fe^{3+} + HO^- + HO^* \quad \text{(Eqn. 3)}$$

$$RH + OH^* \text{ --> } R^* + H2O \quad \text{(Eqn. 4)}$$

$$R^* + Fe^{3+} \text{ --> } R+ + Fe^{2+} \quad \text{(Eqn. 5)}$$

The decrease of available transition metals, concomitant with the oxygenation of the atmosphere, posed a problem for the acquisition of metals required for catalysis. This stimulated some bacterial systems to re-invent metal-mediated catalysis; for example, tungsten was substituted by molybdenum in enzymes of aerobic organisms because Mo is chemically similar and much easier to obtain, but some thermophilic obligate anaerobes, e.g. *Pyrococcus furiosus*, still posses tungsten proteins (Hille, 2002). The widespread abundance of Fe-S clusters is especially remarkable when considering the decline in available iron upon the onset of oxygenation (Anbar and Knoll, 2002), but apparently the biochemical benefits outweighed the high costs of acquiring iron through siderophores or extracellular reductases. Besides the use of metals as catalysts, at some (debatable) point in evolution micro-organisms started to use metals as electron donors (e.g. bacterial iron oxidation) or as final electron acceptors ("breathing" iron- or manganese-oxides) (Weber et al., 2006). These Bacterial interactions with solid phase metals, or their respective oxides and minerals, has intrigued researchers for decades. Research in this area is now an important part of a new field in environmental sciences: geomicrobiology.

Metal toxicity in biological systems

As mentioned, many metals such as calcium, cobalt, copper, iron, manganese, sodium and zinc are required nutrients and essential for the survival of a bacterial cell. Therefore micro-organisms have developed uptake systems for these metals (Worm et al., 2006). These systems may be either "cheap", unspecific and constitutively expressed or relatively costly and very specific. Other metals such as silver, cadmium, lead and mercury lack biological function and are nonessential, but may be taken up anyway via unspecific uptake systems. Examples of undesired influx via unspecific uptake systems are the uptake of arsenate via the fast Pit (phosphate inorganic transport) system and the influx of chromate by sulphate-uptake systems. Both importers recognize their substrate based on diameter and charge, characteristics that are similar for the above mentioned oxyanions, and as a consequence causes the mix-up (Nies, 1999).

Most heavy metals are transition elements with incompletely filled d orbitals, providing heavy metal cations with the ability to form complex compounds. Once in the cell, heavy metals attack various compounds, especially SH groups, therewith causing a variety of toxic effects. Metal toxicity (expressed as minimal inhibitory concentration or MIC) can be roughly predicted by the affinity of a metal for sulphur, as postulated by Nies in 1999. MIC is a measure often used to compare metal-resistance between different species or between different types of media. Basically, micro-organisms are cultured on plates with medium containing different metal concentrations and the lowest concentration where no colonies are found is defined as the MIC (Mergeay et al., 1985). When plotting log(MIC) versus log(KMeS-) a linear dependency is detected for datasets involving both *Escherichia coli* and *Cupriavidius metallidurans* (previously known as *Wautersia, Ralstonia and Alcaligenes*), as shown in figure 1. The latter species is an extremely metal-resistant β-Proteobacterium, that was isolated from a zinc decantation tank in Belgium and subsequently also found in other heavy-metal contaminated environments (Diels et al, 1995; Goris et al, 2001). As can be deduced from figure 1, toxic heavy metals (e.g. Cu^{2+} and Hg^{2+}) bind with higher affinity to intracellular thiol-compounds, than do essential metals (e.g. Mn^{2+} and Co^{2+}) (Nies, 2003). For this reason toxic metals may replace essential metals in their native binding sites in active sites of enzymes or cell walls, thus causing damage by altering structural conformations. Another mode of toxicity is caused by the generation of radicals by Fenton-type reactions. For copper, a process was proposed by Solioz and Stoyanov (2003), which involved the generation of hydroxyl radicals (which attack DNA bases and break phosphodiester bonds), and depletion of the glutathione pool by redox-cycling of copper (thus hindering the incorporation of metals in metallo-enzymes). In addition, toxicity may be due to interference with the intracellular osmotic balance, which normally is regulated by sodium and potassium (Bruins et al., 2000).

At ecosystem level, the toxicity of heavy metals is considerably different from the effects caused by hydrocarbon or carbohydrate contamination. Pollution of soil and aquatic systems with (polyaromatic) organic compounds, e.g. benzene, toluene, naphthalene, TNT etc, are topics covered in many publications of the last decades (Milhelcic & Luthy, 1988; Spain, 1995; Kasai et al., 2005) and show that most contaminants (if bio-available) can be broken down into smaller, less toxic, compounds by a surprisingly large part of indigenous micro-organisms. In the most optimistic scenario, harmless CO_2 and H_2O are the

only end products after microbial degradation of hydrocarbon pollution, whereas heavy metals cannot be broken drown; toxic metals tend to accumulate in different types of organisms or tissues and can give rise to bio-concentration and bio-magnification, especially in aquatic ecosystems and biofilms (Labare et al., 1997; McIntyre and Beauchamp, 2007). Metal accumulation in aquatic organisms results from the net uptake of contaminants from water (bio-concentration) and from food (trophic transfer or dietary accumulation). Well-known examples of toxic accumulation of metals in food webs include mercury, tributyltin and tetraethyl lead.

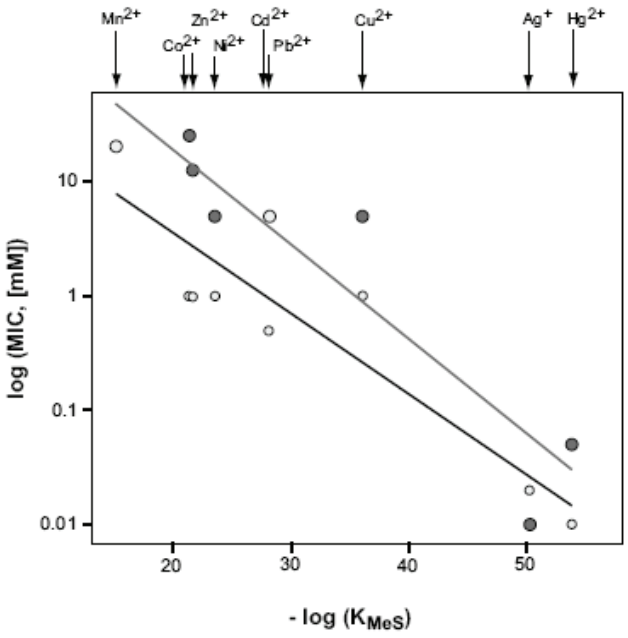


Figure 1: Heavy metal toxicity depends on the affinity to sulphur (adapted from Nies, 1999). The logarithm of the MIC of heavy metal cations for *E. coli* (open circles) and *C. metallidurans* (closed circles) was plotted against the logarithm of the solubility for the respective metal sulphide. The positions of cations are indicated at the top. An exponential curve fit was performed for both data sets that yielded regression coefficients of 81.1% and 86.7%, respectively.

Metal pollution in marine environments

Though toxic heavy metals have been present on Earth since the beginning of life four billion years ago, anthropogenic influences have considerably increased metal pollution level in many types of environments. Metals have been extensively used throughout human history as paints, tools, weapons, poisons and medicine among others; currently, heavy metals still have many applications. Silver compounds continue to be considered best antiseptics in dressings for burns. Also, mercury is used in gold mining in many parts of Latin America, arsenic is still common in wood preservatives, and tetraethyl lead remains an attractive additive to petrol, although this use has decreased dramatically in the developed countries.

As far as the marine environments are concerned, major sources of heavy metal input into the marine environment are industrial discharges, waste disposal streams and atmospheric deposition of exhaust gasses. More stringent environmental legislation has led to a decrease of these metal discharges in the last 15 years (EEA Report, 2003). However, considerable heavy metal pollution of sediments from harbours and marinas subsists and has been attributed to the application of antifouling paints on ship hulls of sea vessels (Schiff et al., 2004; Warnken et al., 2004). Some of these locations need to be dredged frequently for navigational purposes, as is the case for many harbours in North-Western Europe from which more than 200 million cubic meters of contaminated sludge is dredged on an annual basis (Bortone et al., 2004). Dredging operations can increase metal mobilization by whirling up fine sediment particles and allowing oxygen to come in contact with previously buried and reduced sediments. The extent of metal release depends on local parameters such as sediment geochemistry, currents, grain size, pH and salinity (Vale et al., 1998; Van den Berg et al., 2001).

When the level of contamination of dredged sediments is low, risk assessment studies show that in situ capping (confinement by an inert barrier) or passive natural attenuation offer viable alternatives to dredging (Wang et al., 2004). When recycling of metals is desired polluted sediments can be treated microbially in solid bed reactors (Löser et al., 2006), washed with biosurfactants (Mulligan et al., 2001) or subjected to phyto-extraction (Garbisu & Alkorta, 2001). The contaminated material can also be used as landfill, preferably combined with long-term monitoring since in some cases elevated levels of metals can be measured in the leachate and in the groundwater (Kalyuzhnyi & Gladchenko, 2004). Another possible application for polluted sludge is the production of bricks or glass (Poon et al., 2001; Coruh et al., 2006), in which the metals are immobilized. Despite this multitude of options, the most common practice is to simply relocate the dredged material in the same water system, under the assumption that this procedure minimizes changes in biotic and abiotic parameters (Bortone et al., 2004).

Effects of metals on microbial diversity

Heavy metals tend to accumulate in sediments, due to adsorbance to clay particles, humic acids, iron(hydr)oxides, sulphides or organic matter (Glasby & Schulz, 1999; Leipe et al, 2005). Micro-organisms can influence metal availability directly through oxidation/reduction reactions, as well as indirectly by production (or degradation) of acids, sulphides, metal(hydr)oxides and extracellular polymeric substances, as depicted in the scheme in figure 2 (Gadd et al., 2001; Suzuki, 2000; Lloyd & Lovley, 2001; Valls & de Lorenzo, 2002).

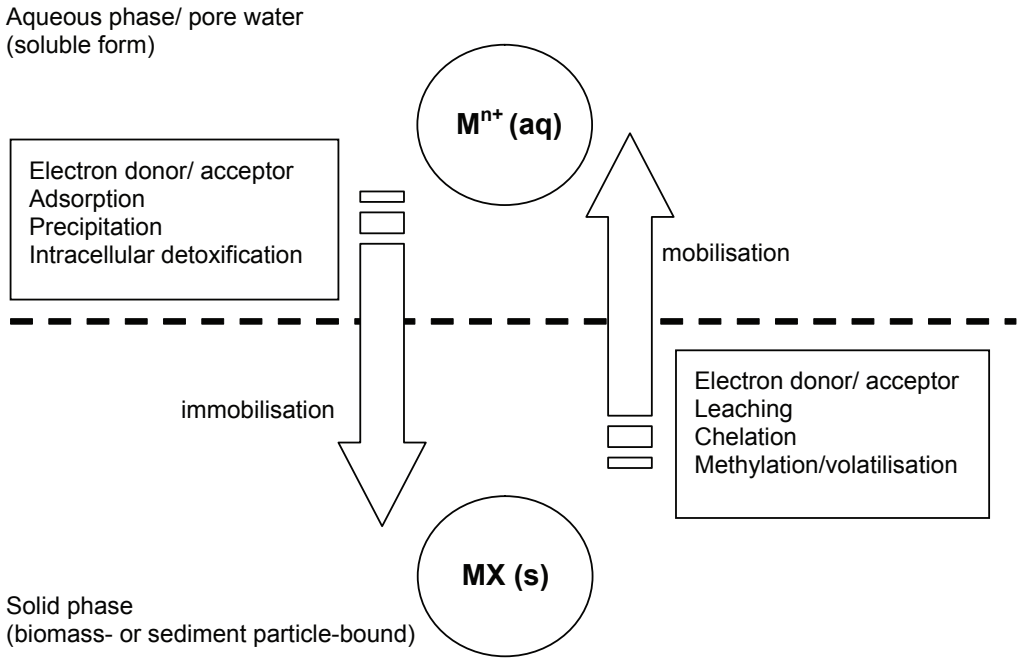


Figure 2: Scheme summarising possible interactions between aquatic or benthic micro-organisms and heavy metals in a sedimentary environment.

Regarding bacterial heavy-metal resistances, extensive research has been done mostly with pure cultures (Silver, 1996; Nies, 1999). Recently however, some insight has also been gained into the genetic flexibility of microbial populations and the wide-spread occurrence of lateral gene transfer (Sobecky et al., 1998; Cook et al., 2001; Coombs and Barkay, 2004). Research on heavy metal impact on bacterial ecology concentrated on terrestrial environments (Roane and Kellog, 1996; Sandaa et al., 1999a) or concerned freshwater environments (Feris et al., 2003). So far, few studies focused on bacterial diversity in marine sediments impacted by heavy metals, i.e. in a Norwegian Fjord (Gillan et al., 2005), in the Baltic Sea (Edlund et al., 2006), in the New Bedford Harbor area, Massachusetts (Sorci et al., 1999) and on the Windmill islands in Antarctica (Powell et al., 2003). Mechanisms of Archaeal metal homeostasis are just starting to become unravelled by pure culture studies and through comparisons across global transcriptional responses to metal stress (Kaur et al., 2006, Remonsellez et al., 2006). Effects of heavy metals on the (methanogenic) Archaeal community are rarely described (Capone et al., 1983, Sandaa et al., 1999b); similarly, publications describing the metal response of (artificial) phytoplankton communities are not frequently encountered (Sugiura et al., 1982).

It is generally believed that heavy metal pollution affects size and diversity of a microbial community, as extrapolated from principles in macro-ecology (Sandaa et al., 1999a; Sandaa et al., 1999b; Worm et al., 2002; Li et al., 2006). However, some studies have also demonstrated that microbial diversity does not necessarily decrease with increasing environmental stress (Powell et al., 2003; Gillan et al., 2005). The two latter studies focused on long-term polluted and pristine sites on Antarctica and a Norwegian fjord, respectively. The authors used molecular methods as 16S rDNA clone libraries and denaturing gradient gel electrophoresis (DGGE) to estimate microbial diversity and noticed shifts in dominant species, while the total number of species remained more or less constant. It seems therefore probable that sensitive species disappear upon contamination, whereas growth of the more resistant types of micro-organisms is stimulated. Besides adaptation, other factors controlling biodiversity may have played a role in these naturally complex systems, e.g. the amount of carbon, the bio-availability of heavy metals, and presence of grazers. For instance, for the Antarctic sites it was shown that the metal contaminated sites also contained more petroleum, which can be an energy source for a number of Bacteria as well as a sorbent for heavy metals (Powell et al., 2003). Gillan and co-authors hypothesized that timescale may be critical, as metal-pollution in the Norwegian fjord under study started as early as 1920; the microbial communities have had over 90 years of adaptation time (2005).

Instead of using the 16S rDNA "household" genes for taxonomic purposes and diversity studies, another approach directly targets functional genes in pollutant or resistant pathways from polluted and pristine sites (Oger et al., 2003; Nyyssonen et al., 2006). These types of studies give information about the capacity of a certain microbial population to cope with environmental pollutants and can provide some insight into the importance of horizontal gene transfer (HGT). For example, Rasmussen and Sörensen measured an increase in the percentage of isolates carrying plasmids with Hg-resistance determinants from 62% to 29%, when comparing polluted with pristine sites, respectively (1998).

Metals as microbial energy source

Geochemical gradients

Water bodies, sediments, aquifers and submerged soils are characterised by stratification or geochemical gradients, which can be of an energetic advantage for some Bacteria and Archaea. The oxidation of organic matter can be coupled to reduction of various inorganic electron acceptors, depending on their electron potential and availability. Vertical profiles starting at the water surface show that different compounds are being depleted successively, e.g. O_2, NO_3^-, MnO_2, $Fe(OH)_3$, SO_4^{2-} and finally CO_2. The scales of these gradients may vary; Fe-reduction may occur at a depth of 10m in a typical freshwater pond or at 120m in the Black Sea. In the case of shallow sediments, iron and manganese reduction may occur within cm's or even mm's of the sediment-water interface.

The contribution of iron and manganese to total microbial carbon oxidation varies depending on local parameters. Although in most anoxic marine sediments sulphate reduction is the predominant type of metabolism, it has been shown that in some locations iron can account for up to 80% of all carbon oxidised (Thamdrup et al., 2000, Jensen et al., 2003). Manganese reduction usually represents a lower contribution to carbon oxidation, in the range of 15% (Canfield et al., 1993). However, exceptionally higher contributions of manganese to carbon turnover have been measured; for instance in Oneida Lake sediments (NY, USA), where the Mn-reducing *S. oneidensis* was first isolated from, estimations accounted up to 40% (Nealson & Saffarini, 1994) and in the Panama basin and Skagerrak sediments manganese may be responsible for 90 to 100% of total carbon oxidation (Nealson & Scott, 2006). Manganese oxidation rates can vary greatly between different environments: 2pM/h in hydrothermal vents, 65nM/h at the oxic/anoxic interface of the Black Sea and 350nM/h in some freshwater ponds (Tebo et al., 2004).

Since the deeper layers of marine sediments are dominated by sulphides it can be assumed that the majority of bio-available heavy metals will be immobilised effectively in this region. Amorphous pyrite (FeS_2) is in fact the most soluble of metal sulphides and therefore the iron atom will be replaced by heavy metals that have a higher affinity for sulphur (Naylor et al, 2006). Biogenic iron- but especially manganese-oxides are also considered important repositories for heavy metals in marine systems due to cation exchange, sorption and redox properties (Lewis & Landing, 1992; Tebo et al, 2004). Because of these complex interactions and lack of proper tools, little knowledge was available on the distribution and dynamics of heavy metals in sediments for a long period of time. With the development of microsensors, special devices for measuring small quantities of metals on a micrometer scale, some of the aspects of trace metal chemistry could be elucidated. For instance, Tankere-Muller and co-authors were able to prove that the mobilization of heavy metals in sediments was primarily linked to the decomposition of organic matter and the associated reductive dissolution of iron and manganese oxides (2007).

Bacterially-mediated metal redox cycling

Although it was known for over a century that some micro-organisms had the ability to reduce iron and manganese, the capacity to sustain growth by using metals as final electron acceptor was first elucidated in the 1980's, when physiological properties of two metal reducing bacteria were described: *Shewanella oneidensis* (Myers & Nealson, 1988) and *Geobacter metallireducens* (Lovley & Philips, 1988). These were the first examples of Bacteria that could conserve energy through the reduction of extracellular, insoluble substrates, as depicted by reactions 6 and 7:

$$Fe(III) + C_{org} \dashrightarrow Fe(II) + CO_2 \text{ (or other oxidized } C_{org}) \qquad \text{(eqn 6)}$$

$$Mn(IV) + C_{org} \dashrightarrow Mn(III)] + C^*_{org} \dashrightarrow Mn(II) + CO_2 \text{ (or other oxidized } C_{org}) \qquad \text{(eqn 7)}$$

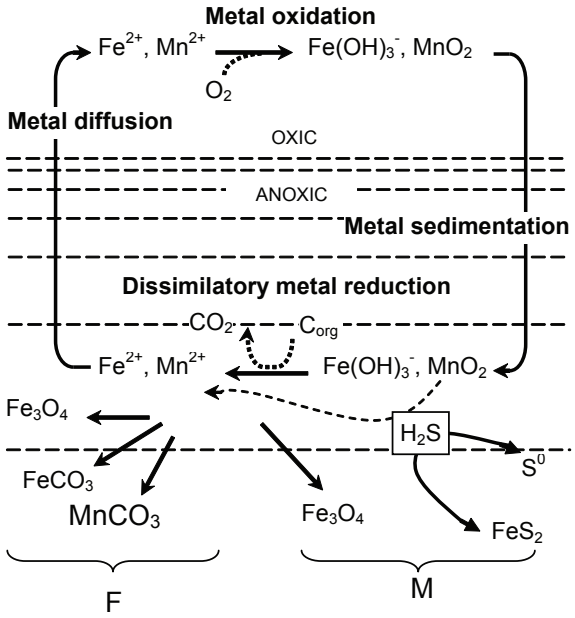
Since then more species with similar abilities were identified, representing phylogenetically distinct groups including both Gram-positive and Gram-negative Bacteria, Euryarcheota (*Archaeoglobus fulgidus, Pyrococcus furiosus*) and Crenarcheota (*Pyrodictium abyssi*) (Lovley et al., 2004; Lloyd, 2003). However, precise mechanisms appear to be more complex than anticipated and possibly comprise excretion of siderophores to solubilize the metaloxides or electrically conductive nanowires in some cases (*Shewanella*) and/or membrane-associated protein complexes with cytochromes, reductases and chaperonnes, suggesting a requirement for actual mineral attachment in other cases (Nealson and Myers, 1992; Nealson et al 2002; Lovely et al, 2004; Gorby et al, 2006).

Besides the fact that iron and manganese belong to the most abundant metals in the Earth's crust, another advantage for micro-organisms utilising these metals as electron acceptor is that they are readily recycled, as depicted in figure 3. Fe^{2+} and Mn^{2+} easily dissolve in water, unlike the insoluble oxide from which they were derived. This increases their mobility and causes an upward diffusion towards the oxic zone, where they may be spontaneously oxidised again, subsequently precipitate and sink. Alternatively, metal oxidation can also be catalysed by microbial interaction, e.g. anaerobic oxidation of ferrous iron can be achieved by phototrophic organisms (e.g. *Rhodovulum*), by denitrifiers or by aerobic Bacteria (e.g.

Thiobacillus ferrooxidans) (Straub et al., 2001). Mn^{2+} oxidation has been observed in several Gram-positives (*Bacilli*), Proteobacteria (*Pedomicrobium, Leptothrix and Pseudomonas*) and even fungi, although the ability to sustain growth with this metabolic trait has not been unequivocally confirmed (Tebo et al, 1997).

Removal of iron and manganese from the biological cycle occurs by precipitation to inactive mineral oxides and subsequent burial into the sediments (see figure 3). When considering a freshwater aquifer, carbonate based minerals and Fe_3O_4 (magnetite) are most likely to be formed. In comparison, seawater contains a wealth of complexing anions in addition to copious amounts of sulphate and manganese. The active sulphur cycle will lead to the production of sulphides and pyrite (FeS_2), precipitating all available iron. Tetravalent manganese can act as an oxidizing agent to chemically oxidize sulphide, producing inorganic sulphur, therewith stimulating sulphate reduction in general.

Figure 3: Scheme showing proposed cycles of iron and manganese and their removal from the system under freshwater (F, bottom left) and marine (M, bottom right) conditions (modified from Nealson and Saffarini, 1994)



Shewanellaceae

The first description of a member of the *Shewanella* was published in 1931 when Derby and Hammer isolated a hitherto unknown bacterium from putrefied butter and named it *Achromobacter putrefaciens*. Ten years later it was suggested by Shewan to change its taxonomic position to the *Pseudomonads*, from where it was removed again in the early 90's to finally receive its own genus residing within the *Vibrionaceae.* The genus *Shewanella* comprises an ever increasing number of metabolically flexible, facultatively anaerobic, rod-shaped micro-organisms found primarily in aquatic habitats. Phylogenetically the *Shewanella* are most closely related to the genera of *Vibrio* and *Photobacterium* within the Gamma-subclass of the Proteobacteria.

Although the first publications focusing on *Shewanella* dealt primarily with their relevant role in fish spoilage and subsequent human infections, the availability of DNA based methods allowed the detection of *Shewanella* species in basically all the world's oceans, the underlying sediments and its inhabitants, as can be seen in table 1. Notably, this bacterium is not restricted to marine waters since *Shewanellae* have additionally been discovered in freshwater environments such as the Amazon shelves and Oneida Lake. Shewanella species were also recovered from environments with varying temperatures and pressure, i.e. from tropical regions to Arctic sea ice and the bottom of the deep sea. The wide spread abundance of these bacteria together with their ability to reduce a whole range of different metals, metalloids and radionuclides (e.g. U, V, Mo, Cr, Co, As, Tc and Se) have made them intriguing research subjects over the last decades. Additionally, the flexible metabolism of *Shewanellaceae* enables these Bacteria to instantly respond to changes in their environment. This feature, combined with their appetite for rich organic matter and the vast array of chemotactic genes found in its genome (Li et al, 2007), make *Shewanellae* excellent opportunists in a gradient-driven world, such as submerged sediments (Nealson et al., 1997).

Table 1: Source of isolation, geographical position, temperature range, salt dependency (% Na), metal-reduction capacities (V indicates variability between strain) and sulphide formation of various *Shewanella* typestrains.

Typestrains:	Source	Geography	T (°C)	Na	Me	H2S	Reference
S. affinis	benthic worm	Sea of Japan, NW Pacific	10- 34	0.5-6	ND	+	Ivanova, 2004
S. colwelliana	oyster						Coyne, 1989
S. loihica	hydrothermal vent	Loihi seamount, Hawaii	0- 42	0.5-5	+++	-	Gao, 2006
S. marisflavi	seawater	Yellow Sea, Korea	4- 42	1-9	ND	+	Yoon, 2004
S. gelidimarina	sea ice	Antarctica	0- 23	1-6	Fe	+	Bowman, 1997
S. benthica	intestine	South Atlantic, depth 11000 m	<25		V	+	Venkateswaran, 1999
S. marinintestina	intest. squid	Yokohoma, Japan	4-30	1-5	ND	+	Satomi, 2003
S. schlegeliana	intest. fish	Hiroshima, Japan	4- 32	1-5	ND	+	Satomi, 2003
S. woodyi	seawater	Alboran Sea, depth 300m	4- 25	1-5	ND	-	Makemson, 1997
S. hanedai	seawater	Sea of Japan	4- 25		-	-	Jensen, 1981
S. amazonensis	intertidal flat	Amazonian shelf sediments	4- 40	1-5	++	+	Venkateswaran, 1999
S. algae	red algae	Sea of Japan	10- 41	1-10	Fe	+	Khashe, 1998
S. putrefaciens	putrefied butter	Lake Michigan, US	4- 32	0-6	++	+	Derby, 1931
S. oneidensis	freshwater sed.	Lake Oneida, US	meso	0-3	++	+	Venkateswaran, 1999
S. baltica	seawater	Baltic Sea	4- 30		V	+	Ziemke, 1998
S. frigidimarina	sea ice	Antarctica	0- 27	0-9	Fe	+	Bowman, 1997
S. denitrificans	seawater	Gotland Deep, depth 130m	4- 30	0-6	-	?	Brettar, 2002
S. sairae	Intest. fish	Pacific Ocean	4-27	1-5	-	+	Satomi, 2003
S. pealeana	Squid gland	Woodshole harbour	4-30	1-4	+	+	Leonardo, 1999
S. violacea	Deep sea sed.	Japan, depth 5000m	2-20	1-5	-	-	Nogi, 1998
S. fidelis	Sediment	South China Sea	4-30	0-8	-	?	Ivanova, 2003b
S. waksmanii	Peanut wurm	Sea of Japan	4-30	1-6	-	-	Ivanova, 2003
S. aquimarina	Seawater	Yellow Sea, Korea	10-42	1-9	-	+	Yoon, 2004
S. psychrophila	Deep-sea sed.	West-Pacific	0-20	1-7	-	-	Xiao, 2007
S. piezotolerans	Deep-sea sed.	West-Pacific	0-28	1-7	Fe	+	Xiao, 2007

Microbial metal resistance

Defence mechanisms against heavy metals

In general, four types of microbial heavy metal defence mechanisms can be distinguished: i) intracellular sequestration, ii) extracellular complexation or precipitation, iii) enzymatic detoxification and iv) outward transport of metals (Silver, 1996; Nies, 1999; Bruins et al., 2000). The first two mechanisms aim to decrease bio-availability of toxic metals, whereas the latter two actually result in net reduction of intracellular free metal concentrations.

Intracellular sequestration is the accumulation of metals by binding or precipitation, which prevents sensitive intracellular components from exposure to the metals. As mentioned previously, metals have an affinity for sulphur compounds and are sequestrated to metallothioneins. Sequestration with thiol-containing compounds is actually a rather expensive way for a cell to remove its accumulating metal ions, when compared to efflux systems or metal reduction and is therefore rarely detected. In order for an aerobically growing cell to detoxify Cd^{2+} by producing CdS 16 ATP are required, in stead of 1 ATP needed to actively extrude the metal ion from the cytoplasm with a transporter (Nies, 1999). If glutathione or a metallothionein are considered, investments are truly enormous. A bacterial cell need only deploy such a detoxification system when concentrations of heavy metals are low, or when a futile cycle of metal uptake and export needs to be disrupted. Although often encoded in genomes of higher eukaryotes, only one example of prokaryotic metallothionein has been described thus far, i.e. smtA by *Synechococcus* sp. SmtA can potentially bind up to four zinc atoms with cystein residues and its transcription is induced in presence of high levels of Cd(II), Zn(II) and Cu(II) (Cavet, 2003). Three low weight, cystein-rich proteins are produced by *P. putida*, which may also be involved in intracellular cadmium sequestration (Bruins et al., 2000).

Occurrence of extracellular sequestration, i.e. the excretion of compounds with the ability to form complexes with metals, has so far primarily been observed in yeast and fungi. *Saccharomyces cerevisiae* reduces the absorption of nickel to cell components by excreting glutathione and copper-tolerant fungi excrete oxalate. Contrastingly, bacterial membranes seem to respond to toxicity by enabling sorption of heavy metals to their extracellular matrix. An example is provided by *Klebsiella aerogenes*, producing a polysaccharide capsule upon copper stress which binds copper very efficiently (Cha & Cooksey, 1991) and excreting sulphide-containing compounds in order to precipitate PbS, CdS and even HgS (Aiking et al., 1985). A slightly different mechanism was demonstrated in *Pseudomonas putida*, which immobilised large amounts of Cd(II), Cu(II) and Zn(II), through sorption to the extracellular matrix (Chen et al., 2005). Intriguingly, purified exopolysaccharide by itself appeared not as efficient in Cd(II) binding as an organism with intact extracellular coating (Bruins et al., 2000). *Citrobacter* sp. displays the ability to form insoluble complexes as $CdHPO_4$ or $PbHPO_4$ (Puranik & Paknikar, 1999). Uncertainties remain about whether these responses are of a biological nature or whether it is a chemical phenomenon. Indeed, results of Mergeay and co-authors indicated that such metal accumulation, which can account for up to 15% of bacterial dry weight, was related to progressive alkalinisation of culture medium and accompanied by metal precipitation as carbonates or hydroxides (1991).

More common, but highly specific for certain metals, is enzymatic detoxification of metals to less toxic forms. Reduction of metals is only energetically favourable when the redox potential of the respective metal is between the hydrogen/proton couple (-421 mV) and the oxygen/hydrogen couple (+808 mV) at physiological conditions (pH 7 and 30°C). Only few heavy metals meet these requirements, e.g. mercury, chromate, arsenate and copper (Nies, 1999). Additionally, the cell needs to be able to get rid of the reduced metal compound, sometimes a cause for problems in the cases of Cr^{3+} (quite insoluble) or AsO^{2-} (more toxic than AsO_3), requiring specific transporters. By far the best studied bacterial detoxification mechanism is the removal of mercury, involving the enzymatic reduction of Hg^{2+} to the relatively harmless Hg^0 which spontaneously diffuses to the atmosphere (Rasmussen & Sörensen, 1998; Barkay et al., 2003). Reduction of arsenate leads to the more toxic arsenite, but it also enables the cell to distinguish between arsenite and phosphate and subsequently specifically export arsenite (Silver, 1996). The enzymatic machinery involved in arsenate resistance has additionally been held responsible for the detoxification of antimony in some cases (Nies, 1999). Methylation, i.e. addition of a methyl group, gener-

ates metallic substances that are (usually) more volatile, a feature that enables Bacteria to easily remove metal compounds from the cell. Methylation of metallic elements has been shown to be part of microbial detoxification strategies, for instance in the case of mercury, lead, arsenate, antimony, cadmium, bismuth and tin (Gadd, 1993; Gadd et al., 2001). On the other hand, Drott and co-authors state that mercury methylation is an accidental process, promoted by passive uptake of neutral Hg species over the cell membrane of anaerobic methylating bacteria (2007). Biological methylation and subsequent volatilisation has been suggested to exist for more exotic elements as well, such as Se, Te, Ge and Tl (Donard & Weber, 1988).

Last of the different resistance mechanisms is also the most common in bacteria and perhaps most effective: removal of toxic metals from the cytoplasm or periplasm by active transport or efflux systems. Indeed, when the influx of heavy metals cannot be hindered (or is too costly at long-term), it seems most logical to focus all efforts on pumping the metals out of the bacterial cell.

Heavy metal efflux pumps

So far, there are three major groups of heavy metal efflux pumps: CDF proteins (cation diffusion facilitator), CPx-type ATPases and RND proteins (resistance nodulation cell division). These protein pumps are representatives of very divergent protein super families (Paulsen et al., 1996; Coombs & Barkay, 2004, 2005; Saier, 2000), suggesting that the need to protect cells from heavy metal toxicity existed early in evolution. The first experimental evidence for the importance of heavy metal pumps for metal-resistance was provided by genetic studies on the before mentioned *C. metallidurans*. This bacterium harbours two megaplasmids, designated pMOL28 and pMOL30, encoding metal efflux pumps for nickel, cobalt, cadmium and zinc among others. Loss of one or both plasmids greatly increased metal sensitivity, as is shown in table 2 (Mergeay, 1985). More recent investigations have further elucidated the importance of these efflux systems for basic levels of metal tolerance in several other bacterial species (Nies, 1999; Legatzki et al., 2003a, 2003b; Munkelt et al., 2004). Moreover, newly annotated genomes indicate an abundance of genes (putatively) encoding heavy metal efflux pumps in deep-branching types of Bacteria, Archaea and Eukaryotes, strengthening this assumption (Canovas et al., 2003; Baker-Austin et al., 2005; Barton et al.,, 2006; Monchy et al., 2006; Ettema et al., 2006). Table 3 provides an overview of the general characteristics of three types of heavy metal efflux pumps, which are discussed in more detail below.

The first type of metal efflux pumps belongs to the RND proteins, including examples as the czcCBA-system of *C. metallidurans*, exporting cobalt, zinc and cadmium, and the cusCFBA-system in *E. coli*, responsible for transportation of monovalent copper and silver (Nies, 2003). The majority of genes encoding RND proteins in Proteobacteria, is located adjacent to gene(s) encoding a membrane fusion protein and an outer membrane factor. Microbial RND proteins that have been studied elaborately because of their medical importance belong to the so-called multidrug exporters, responsible for antibiotic resistance and possibly also involved in virulence (Piddock, 2006). The only available crystal structure of an RND protein is from the multidrug exporter AcrB from *E. coli*. Biochemical evidence and x-ray analyses indicate that this protein contains 12 transmembrane regions, spanning the inner membrane, periplasmic space and outer membrane. Transport occurs in an antiport fashion, i.e. at the cost of proton motive force (Paulsen, 1996). If characteristics of the antibiotic RND proteins can be extrapolated to the metal pumps, these are probably also able to take up metal cations from cytoplasm, cytoplasmic membrane or periplasm while extruding them to the cells exterior (Goldberg et al., 1999).

Metal	MIC[1] (mM)			
	CMwt[2]	CM3	CM4	CM5
Ni^{2+}	2.5	0.6	2.5	0.6
Zn^{2+}	12	12	0.6	0.6
Cd^{2+}	2.5	2.5	0.6	0.6
Co^{2+}	20	20	5.0	0.07
Hg^{2+}	0.7			0.25

Table 2: Summarised data from toxicity tests withdifferent heavy metals to C*upriavidius metallidurans* (CM) and its plasmid free derivatives.

[1] MIC (minimal inhibitory concentration) for *Cupriavidus metallidurans* determined as described in Mergeay, 1985.
[2] wildtype strain CH34, CM3 w/o pMOL28, strain AE128; CM4 w/o pMOL30, strain AE126 and CM5 plasmid free strain AE104.

Table 3: Overview of general characteristics of three types of heavy metal efflux pumps: heavy metal RND proteins, CDF proteins and CPx-type ATPases (Nies, 1999; Silver, 2003, and the transport classification database at www.tcdb.org).

Type	Energy[1]	Size[2]	Examples[3]	Metals	Organisms
RND	pmf (H^+)	110 (12)	CusA (EC)	Cu^+/Ag^+	Mostly Gram- Bacteria
			CzcA (CM)	$Co^{2+}/Cd^{2+}/Zn^{2+}$	
			SilA (ST)	Ag^+	
			CnrA (CM)	Ni^+/Co^+	
CDF	pmf (H^+, K^+)	36 (6)	ZitB (EC)	Zn^{2+}/Cd^{2+}	All organisms
			YiiP (EC)	$Cd^{2+}/Zn^{2+}/Fe^{2+}/Hg^{2+}$	
			czcD (CM)	$Co^{2+}/Cd^{2+}/Zn^{2+}$	
			czcD (BS)	Co^{2+}/Cd^{2+}	
			ZntA (SA)	Co^{2+}/Zn^{2+}	
CPx	ATP	80 (8-10)	CadA (SA)	$Pb^{2+}/Cd^{2+}/Zn^{2+}$ (efflux)	All organisms
			CadA (HP)	$Co^{2+}/Cd^{2+}/Zn^{2+}$ (efflux)	
			CopA (EC)	Cu^+/Ag^+ (efflux)	
			CopA (EH)	Cu^{2+} (uptake)	
			CopB (EH)	Cu^+/Ag^+ (efflux)	
			SilP (ST)	Ag^+ (efflux)	

[1] Energy refers to the costs of transport (proton motive force (pmf), charge, pH or ATP).

[2] Size lists the approximate weight of the protein in kDa and the number of transmembrane helices between brackets.

[3] EC is *E. coli,* CM is *C. metallidurans* and ST is *S. typhimurium*, BS is *B. subtilis*, SA is *Staphylococcus aureus*, HP is *H. pylori*, EH is *E. hirae*.

The second category of metal resistance proteins comprises CDF proteins. These proteins are found in all three domains of life, mediating transport of metal cations making use of chemiosmotic gradients, potassium, charge or pH gradients. In general, CDF proteins are relatively small (300-400 amino acids) and distinguish their substrate based on size and binding-affinity. Most CDF proteins studied so far transport only divalent cations with ionic radii of around 74±2 pm, i.e. iron, zinc, cobalt, nickel, manganese and cadmium (Nies, 2003). One example of a CDF protein in *E. coli* is ZitB, which diminishes cellular zinc accumulation at the costs of a potassium gradient in addition to the proton motive force.

The third type of heavy metal pumps consists of P-type ATPases, a superfamily of transport proteins that are driven by ATP hydrolysis. P-type ATPases can either import (from periplasm to cytoplasm) or export substrates (from cytoplasm to periplasm/outside) and are, due to this duality, often important determinants in heavy metal homeostasis. P-type ATPases usually carry inorganic cations as H^+, Na^+, K^+ and Ca^{2+} across a membrane. Members of this family that specifically transport heavy metals are called CPx-type ATPases because of the conserved cystein-proline motif, essential for metal-binding (Fan & Rosen, 2002). Heavy metal ATPases span the cytoplasmic membrane with 8 helices and contain several other highly conserved amino acid residues involved in substrate binding and ATP hydrolysis. CadA from *S. aureus* was the first protein from this category to be fully characterised and was shown to transport Zn^{2+}, Cd^{2+} and Pb^{2+} out of the cytoplasm (Tsai et al., 2002). Other relevant examples are involved in export of Cu^+/Ag^+ (copA) or Zn^{2+} (ZntA) (Rensing et al., 2000; Banci et al., 2003; Gaballa & Helmann, 2003). CopA-like transporters are found frequently in *E. coli, Synechocystis, Archaeoglobus fulgidus, B. subtilis, E. hirae, Streptococcus mutans* and lower eukaryotes as *Cryptosporidium parvum* and *Candida albicans* as well as mammals (Ettema et al., 2006; Coombs & Barkay, 2005).

Heavy metal determinants in microbial genomes

Genes encoding single subunit CPx-type ATPases and/or CDF proteins are widespread in bacterial genomes, while the more complex RND proteins are relatively rare. Thus, CPx-type ATPases and/or CDF proteins are generally part of homeostatic systems, while the low frequency of RND proteins suggests that these provide an extra layer of protection (Nies, 2003). The first RND systems that were studied were transporters for organic substances, such as antibiotics and drugs (e.g. AcrB and MexB). The multidrug resistant *Pseudomonas aeruginosa* for instance, carries 12 putative RND systems, which are probably used to mitigate various types of antibiotics. Although RND proteins are not the most frequently found heavy metal determinants, genome comparison showed that RND transport systems are still quite ubiquitous among Bacteria, especially Proteobacteria but also in early-branching species such as *Aquifex aeolicus* and *Thermotoga maritima*. In Archaea, RND sequences were not found and Gram positive bacteria carried fewer copies of the RND proteins or none, which has to be related to their different cell wall chemistry which is less susceptible to heavy metal traffic. With the advent of genomics and improvements in bio-informatics, an increasing number of RND proteins and CPx-type ATPases is being identified, although in most cases experimental evidence for their involvement in metal homeostasis is still lacking.

Marine plasmids, transposons, integrons, bacteriophages and other mobile genetic elements (and their encoded genes) are all members of the horizontal gene pool, which is believed to be of great importance for evolution and adaptation to new environments. Horizontal gene transfer (HGT) provides a variety of mobile genes and recombination properties that may serve for reshuffling, acquisition and exchange of genetic material (Sobecky, 2002). As mentioned, many heavy metal resistance genes are present both on chromosomes and plasmids. In fact, essential metal resistance systems which often are very complex are to a great extent encoded on chromosomes, while simpler systems such as efflux pumps usually are plasmid-encoded (Bruins et al., 2000). A number of findings suggest that spreading of genes with certain phenotypic traits and adaptation of micro-organisms to polluted environments is mediated by HGT. E.g. higher frequencies of plasmid DNA and higher resistance levels against antibiotics and toxic chemicals were observed close to a point where pharmaceutical and industrial wastes were deposited. Furthermore, plasmid transfer to indigenous bacteria has been demonstrated after addition of bacteria carrying conjugative plasmids to marine soil. This phenomenon has been shown to occur at various limiting conditions, such as nutrient depletion, pH and temperature variations (Sobecky, 2002).

16S rRNA genes provide important information about vertical relationships between species. When the phylogeny of a gene differs from the phylogeny of a marker gene such as 16S rRNA, this is called phylogenetic incongruence. Phylogenetic incongruence, together with features such as unusual GC-content, shared patterns of insertions and deletions and the presence of adjacent genes on an operon are considered pointers towards (or evidence for) HGT. Coombs and Barkay (2005) performed a comparative genome analysis on CopA ATPases, in which they found several occasions of such HGT evidence. Upon comparison between the genomes of the extreme metal-resistant *C. metallidurans* and it's more susceptible relative *C. solanacearum*, a higher copynumber of CDF, RND and CPx-type proteins was observed (Nies, 2003). Since most of these proteins show some similarity, it is hypothesised that the increase in gene number is primarily caused by gene duplication. However, two RND proteins from *C. metallidurans* that are located on plasmids show little similarity to homologues in *C. solanacearum*, and were probably acquired through horizontal gene transfer. Not only does *C. metallidurans* carry a higher number of metal-resistant determinants, some of its Zn-CPx-type ATPases have evolved even further and changed their substrate affinity to additionally transport lead and cadmium.

In an environment contaminated with heavy metals, the balance between metal importers and exporters becomes very crucial. Regulation of metal-resistant genes is an intricate business and not many mechanisms have been fully elucidated so far. In the simplest scenario, transport is regulated by a two-component (sensor-effector) mechanism, in which the corresponding metal cation represses transcription (Fur operon in *E. coli* for iron transport) or activates transcription (Pho operon for phosphate in *E. coli*). In addition, chaperone proteins and siderophores may be produced to enhance uptake. Complementary regulatory genes can be located upstream, downstream or on another chromosome all together and is really dependent on the type of organism and the heavy metal.

Scope and outline of this thesis

The present PhD thesis was initiated and funded by the 5th framework directive of the European Union (EVK-CT-2002-00081). This multidisciplinary project focused on the transport, reactions and dynamics of heavy metals in marine sediments (acronym: TREAD), with particular interests in the effects of dredging and re-deposition of metal-polluted sediments. My role in the project was to investigate the microbiological details, in other words: to determine what the effects were of heavy metals on microbial diversity in marine sediments and what effects micro-organisms could have on heavy metal dynamics.

Prior to determining the effects of heavy metals on benthic microbial populations, it was essential to conduct control experiments providing information on the undisturbed community and on the reproducibility of applied methods. Therefore, this general introduction is followed by a chapter dedicated to this preparatory research. Here the reproducibility of molecular fingerprinting techniques, i.e. DNA extraction and subsequent PCR-DGGE analyses of microbial communities in four different marine sediments in small-scale mesocosms is discussed. We show that similarity between DGGE patterns of 3 random surface samples, representing cyanobacterial, archaeal and bacterial populations, was high when concerning silty sediments, and a sand-silt mixture, but less in samples from sandy sediments. In addition, large-scale mesocosms filled with sediments from a metal-polluted harbour basin (silty) and from an intertidal flat (sandy) were employed to study heterogeneity of bacterial communities in horizontal and vertical direction. Besides a basic identification of potentially relevant members of the Bacterial communities in both sediments, DGGE profiles of samples taken at 2mm depth-interval revealed fine-tuned changes in bacterial populations in the silty sediment, which were almost undetectable at 5 and 10mm resolution.

The next chapter discusses the effects of deposition of a 3mm layer of metal-polluted harbour sludge on a relatively undisturbed sandy sediment (10cm) maintained in large mesocosms. Geochemical analyses showed an initial increase in pore water metal concentrations (i.e. iron, copper and cadmium), which subsided after three months of incubation. No influence of the deposited sediment was observed in temporal DGGE profiles of bacterial 16S rRNA genes, while a minor, transient impact on the archaeal community was depicted. Phylogenetic analyses of bacterial 16S rRNA clone libraries showed abundance of members of the *Flavobacteriaceae*, the α- and γ-Proteobacteria, in both the impacted and control sediments. Despite the finding that particular clones of the metal-impacted, sandy sediment clustered together with representatives from the harbour sediment, statistical analyses showed that the two community compositions were significantly different. Nevertheless, a prolonged modification of the original population was suggested by the high fraction Cu- and Cd-resistant bacteria among isolates from the impacted sediment compared to results from the silty and sandy and control sediments.

Hypothesizing that substances as HFO and δ-MnO_2 could represent important heavy metal reservoirs in marine systems, enrichments for Fe(III)- and Mn(IV)-reducing were inoculated with extracts of the metal-polluted harbour sludge and the undisturbed sandy sediment. Three isolates were obtained and according to analysis of 16S rRNA and gyrase B genes, all belonged to the genus *Shewanella*. Chapter four focuses on characterization of the metal-reduction capacities and heavy metal resistance of these three isolates under different growth conditions. Results were compared to obtained data for typestrain *S. oneidensis* MR1. Under aerobic growth conditions the two iron-reducing isolates, FB18 and FS8, were most tolerant to high levels of cobalt and zinc, whereas strain MR1, and particularly isolate MB4, showed more resistance to copper and cadmium. Copper resistance was further investigated under anaerobic conditions with lactate as carbon source and different electron-acceptors, i.e. fumarate, HFO and δ-MnO_2. Tolerance levels of isolates MB4, FB18 and strain MR1 were approximately similar with fumarate, compared to aerobic conditions, but FS8 showed increased susceptibility to copper. With a bi-phasic experimental design, we showed that copper contamination of δ-MnO_2 with amounts above and below maximal adsorption capacity, either decreased Mn(IV)-reduction by MB4 or only affected subsequent growth with fumarate, respectively. When using no or low amounts of copper and under conditions of full microbial reduction, highly crystalline, lemon-shaped particles were produced, in contrast to the amorphous manganese oxide offered initially as electron-acceptor. Although metal-tolerance of these isolates and type-strain *S. oneidensis* MR1, does not compare to typical metal-resistant bacteria as *C. metallidurans*, levels are in the range of pore-water concentrations measured in the mesocosms. Furthermore, results indicated that metal-reducing bacteria could potentially impact dynamics of heavy metals by reductive dissolution.

A closer look at the annotated genome of *S. oneidensis* MR1 revealed the presence of only a selected number of genes known to be involved in copper homeostasis. Novel primers were designed to amplify small nucleotide fragments genes encoding two particular copper exporters: copA, an ATPase with a heavy metal associated domain and cusA, a two channel heavy metal efflux pump of the RND family. Chapter five focuses on applications of these primers, which were initially used to monitor expression of *copA* and *cusA* in RNA extracts of MB4 and MR1 during growth under varying conditions. Additionally, the primers were tested on DNA extracted from microbial communities in sediment. This is described in chapter six. These experiments aimed to correlate the presence of bacterial copper exporters in sediment DNA with copper concentrations. The primers were tested on DNA from a range of North Sea sediments, differing in grain size, salinity, and metal contamination. Results from Q-PCR analyses of *copA*, *cusA* and 16S rRNA genes indicated that the strongest correlation was found between copy number *cusA* and total extracted copper, but only when sandy sediments were excluded. For *copA* no significant correlation with copper was found. Further analysis of microbial DNA extracted from sediment samples taken along a depth profile (5mm interval) in the HB sediment, suggested that *cusA* and *copA* copy numbers were not uniformly distributed with depth, nor correlated with pore water copper concentrations or total extracted copper. The final chapter discusses the results from the previous chapters and provides final conclusions of this thesis.

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Depth profile of intertidal flat sediment consisting of coarse sand

2 Reproducibility of PCR-DGGE and spatial heterogeneity of microbial communities in marine sediment mesocosms

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Abstract

Prior to determining the effects of heavy metals on benthic microbial ecosystems, it is essential to conduct control experiments, providing information on the undisturbed community. In this study, the reproducibility of the polymerase chain reaction followed by denaturing gradient gel electrophoresis (PCR-DGGE) was investigated and conducted on DNA extracts of four marine sediments maintained in different mesocosms. Secondly, heterogeneity of microbial communities in both horizontal and vertical direction was examined. Random surface samples of small-scale mesocosms (~6 litres) provided reproducible DGGE patterns for bacterial, archaeal and cyanobacterial communities for the silty sediments used in this study (HA and HB) and for the sand-silt mixture (MA). The sandy sediment (IF), however, showed more spatial heterogeneity. In the large-scale mesocosms (~72 litres), heterogeneity in both sediments (HB and IF) was evident from visual observation and confirmed by DGGE profiles of selected samples. Thorough analysis of bacterial PCR-DGGE patterns of samples in both horizontal and vertical direction, and subsequent phylogenetic identification of individual bands, showed that the majority of detected bacteria were related to the α- and γ-subclasses of Proteobacteria or associated with the *Bacteroidetes*. Furthermore, this study showed that small changes in microbial populations with depth may be overlooked when using a 1cm sampling resolution. At the 0.5cm resolution, the surface sediments clearly deviated from the lower regions, whereas at 2mm resolution even more changes in microbial diversity could be observed

Introduction

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In environmental research, the objective is often to establish the effect(s) of a disturbance on an ecosystem, and therewith the need arises for methods that allow comparison of biodiversity between pristine and impacted sites. In microbial ecology, this question also frequently plays a role and consequently techniques have been developed to estimate both the number of species and their relative abundance. By widespread application of molecular tools based on the 16S rRNA gene, it has been recognized that diversity in environmental samples is beyond general expectation and that the majority of organisms frequently detected belong to the "yet-uncultured" micro-organisms. In order to monitor ecological changes however, knowledge on individual species is not strictly needed. Therefore, community fingerprinting techniques as PCR-DGGE (polymerase chain reaction - denaturing gradient gel electrophoresis) have gained in popularity as these techniques provide relatively simple methods to create a snapshot of diversity and enable comparison between different samples (Muyzer et al., 1993; Murray et al., 1996; Casamayor et al., 2000; Bowman, et al., 2003).

This research focuses on the effects of heavy metal pollution on microbial diversity in marine sediments. One of the major challenges in determining the structure of microbial communities in submerged sediments is the heterogeneity of such systems (Fenchel, 1996; Sievert et al., 1999; Musat et al., 2006). Due to active aerobic carbon cycling at the surface and sulphate reduction and methane production in lower regions, steep vertical gradients may exist at very small scales. In addition, differences in the horizontal direction may exist because of the basic natural variability in structures of sediments; waves and currents create unevenness on the surface of the sediment, through channels, some deeper parts may become aerated, while others remain anaerobic, and shells or stones may alter the flow or create a barrier for diffusion.

In theory, a great number of microbial niches are conceivable in such an ecosystem and therefore many samples are necessary for a reliable survey (Grundmann, 2004). In order to enable frequent and spatially accurate sampling, and to limit the number of uncontrollable environmental factors, it was decided to use mesocosms for these contamination studies. Mesocosms, or flumes, are glass incubation chambers containing submerged sediment samples with controlled water flow, pH, light, temperature, nutrients, i. e. mimicking natural conditions as good as possible. Mesocosms of varying size and complexity have been used previously to study microbial communities under ambient conditions (Frischer et al., 2000; Riemann et al., 2000), and have also proven valuable in contamination studies (Schwinghamer, 1988; Arias et al., 2003; Hendrickx et al., 2005). However, in order to validate mesocosms as models for natural ecosystems, reproducibility assessments are required (both spatial and temporal). Despite the widespread use of mesocosms, this subject has so far received little attention in literature (Roeselers et al., 2006).

One of the objectives of this research was to investigate the effects of deposition of metal-polluted harbour sludge on benthic microbial communities. In order to do such contamination experiments properly, it was deemed essential to establish a firm base of knowledge on the structure of the existing microbial populations, prior to introducing the disturbance. The experiments described here investigated the reproducibility of the applied molecular tools and attempted to characterize the benthic microbial populations under "natural" conditions (e.g. undisturbed, yet artificially contained). Two different set-ups were used in this study, i.e. eight small-scale mesocosms (~6 litre) filled with four types of sediments and four large-scale mesocosms (~72 litre), containing two of the sediments used previously. Initially, a random horizontal sampling scheme of surface sediments from the small-scale mesocosms was performed to test reproducibility of the DNA extraction method, PCR efficiencies and DGGE-profiling. In the second part of this paper, heterogeneity of microbial populations in the large-scale mesocosms was studied, starting with a global survey of visually distinct sediment samples followed with DGGE depth profiles at different levels of resolution. Concluding, phylogenetic identification of a selection of DGGE bands is presented, as this information proved of structural importance.

Experimental procedures

Mesocosm design and monitoring

Four types of marine sediments from different locations in North-western Europe were used in the first part of this study, e.g. HB and HA, both sediments from industrial harbours, MA, from a small-scale marina and IF, from a relatively undisturbed intertidal flat. These four sediments were maintained in duplicate small-scale mesocosms (15x20x20cm, ~6 litres) fed with artificial seawater (super-soluble sea-salt Biosal, Aqualine Buschke, Berg, Germany) and subjected to natural temperature and light variation for two months during a mild winter (4°C average temperature), located in a greenhouse at the Botanical Garden, Delft University of Technology, Delft, the Netherlands.

Large-scale mesocosms (120x30x20cm, ~72 litres) were constructed and maintained for a period of 18 months at the Max Planck Institute for Marine Microbiology in Bremen, Germany, and filled with sediments from sites HB and IF. These large-scale mesocosms are described in more detail elsewhere (chapter 3). But shortly, they were maintained in a 19°C room with artificial light cycles and constant flow of natural seawater, monitored by measuring nutrients, metals, sulphide and oxygen in the sediments on a regular basis. Besides measuring heavy metals in the pore water fraction (DET probes, results not shown here), conventional metal extraction was performed on ~3g wet sediment, dissolved into 20 ml 0.01M HNO_3, shaken for 24 hours, filtrated through 0.45μm filter and ten fold diluted for ICPMS analysis (Zhang and Davison, 1995).

Sampling strategy

For the small-scale mesocosms, a horizontal sampling scheme was applied to the surface sediments, by sampling three squares of $1cm^2$ that were selected from 248 possibilities by a random number generator (Microsoft Excell, excluding outermost squares); A fourth sample comprised a mixture of the three individual samples. Samples were taken with cut-off syringes and the top two cm of sediment was homogenized before sub-sampling for DNA extraction.

For the large-scale mesocosms, sediment was sampled with syringes as described above and sliced sediment cores were used for depth analysis. Except for the 2mm-resolution depth profile, that involved an innovative combinatory sampling approach; As DGT probe devices were regularly deployed into mesocosm sediments, measuring sulphide and trace metals (Zhang and Davison, 1995), sheets of cellulose filter protecting the underlying sensitive resin, were removed after sediment incubation, carefully sliced in 2mm sections and the filter with adhering bacteria was used for DNA extraction. This method enabled geochemical analyses and microbiological sampling on the exact same location.

DNA extraction and quantification

Extraction of genomic DNA was performed on sediment samples with the Ultra Clean Soil DNA Isolation Kit (MoBIO Laboratories, USA) according to the manufacturer's manual. Typically homogenized sediment samples of 0.25-1.5g were used for extraction. Quality check and quantification of total genomic DNA was done with the Nanodrop ND 1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) or with the PicoGreen assay (Molecular Probes Inc., Eugene, OR, USA).

PCR-DGGE

All PCR reactions were conducted in 50 ml reactions with approximately 50ng target DNA using the Taq PCR Master Mix kit (QIAGEN, Germany), in a Thermocycler (BioMetra, Germany). PCR products were analyzed by gel electrophoresis (1% [w/v] agarose gel, 30minutes, 100V), stained with ethidium bromide and visualized with the BIO-Rad Gel Doc 1000 under UV illumination. Bacterial DGGE fragments were amplified as described previously with primers 341F-GC and 907R (Schäfer and Muijzer, 2001). DGGE fragments from Cyanobacteria and chloroplasts were amplified with primer 359F-GC and an equimolar mixture of the reverse primers 781R(a) and 781R(b), and PCR conditions as described by Nübel et al.

(1997). DGGE fragments of Archaea were amplified with primers Parch519F and ARC915R-GC, and PCR conditions as described previously (Coolen et al., 2004). Overall, when PCR proved difficult due to co-extracted, enzyme-inhibiting compounds, such as humic acids, additives were applied in concentrations that were optimized for each sediment, e.g. additional $MgCl_2$ (up to 4.5 mM), bovine serum albumine (BSA, 0.08-1 µg/µl) or dimethyl sulfoxide (DMSO, 0.5-2 µg/µl).

DGGE was performed as described previously (Schäfer and Muyzer, 2001). Per lane 400ng-1µg of PCR product was loaded, but for comparative reasons each lane within one DGGE gel contained approximately equal amounts of PCR products. For the analysis of bacteria and cyanobacteria, gels with denaturant gradients of 20-80% were run for 16h at 100V. Archaeal communities were analyzed by running gels with a 20-60% denaturing gradient for 5h at 200V.

Phylogenetic analysis

Excised, re-amplified and purified DGGE bands were sequenced with the appropriate forward primer, lacking the GC-clamp. All DNA sequencing reactions were carried out on an ABI 3730 sequencer (Applied Biosystems, USA). Sequences were compared to sequences stored in GenBank using the BLAST algorithm (Altschul et al., 1990; www.ncbi.nlm.nih.gov/BLAST) and subsequently imported into the ARB software program (Ludwig et al., 2004; www.arb-home.de). Alignments were corrected manually when necessary. A phylogenetic tree was constructed using close relatives from the ARB database and imported sequences from GenBank. DGGE sequences were individually imported into this tree and Aquifex aeolicus was used as root. Sequences were deposited at GenBank under accession numbers EU140509 to EU140546.

Results

Sediment characteristics

Initial experiments focused on 4 different marine sediments from North-Western Europe. Typical distinguishing characteristics as grain size and degree of metal contamination are summarised in Table 1. The sediments from the industrial harbour (HA and HB) were comprised of a fine grained, silty material with strong cohesive characteristics. The sediment from the intertidal flat (IF) consisted of well-sorted, coarse sand, as is typical of a high-energy environment. The sediment from the marina (MA) represented a mixture of both sand and silt and showed the highest metal contamination in the solid fraction.

DNA extraction yields

Table 2 summarises the results of 64 individual DNA extractions from marine sediments, with each column representing values for eight individual extractions. According to the manufacturer's protocol, 0.25-1g of wet sediment is the appropriate amount of starting material for optimal extraction efficiency. In initial tests approximately 1g of 4 different sediments (HA, HB, IF and MA) was used, whereas in a later stage the amount of sediment was decreased to ~0.25g of wet sediment (only HB). Sediments containing sand (IF and MA) showed substantially lower yields when compared to the silty sediments (HB and HA). As can be seen from the average DNA content, calculated as the DNA concentration divided by the weight of sediment used, the yield of DNA extraction was considerably improved by applying smaller amounts of sediment. It should also be noted however, that the standard deviation remained considerable.

Table 1: Sediment characterisation

Name	Type location	Grain size	Cu (mg/kg) (SD%)[a]	Cd (mg/kg) (SD%)[a]	Zn (mg/kg) (SD%)[a]
HA	Industrial harbour	Fine silt	0.044 (0.09)	0.0005 (5.44)	0.33 (0.76)
HB	Industrial harbour	Fine silt	0.103 (0.94)	0.006 (4.73)	3.13 (0.42)
IF	Intertidal flat	Coarse sand	0.026 (5.27)	0.007 (3.42)	0.88 (4.23)
MA	Marina	Sand and silt	1.44 (0.70)	0.13 (0.13)	28.4 (1.19)

[a] SD is the standard deviation between three replicate measurements in percentage (%)

Table 2: Yield of DNA extractions from different sediment samples and sample sizes

DNA content (ng/g)[a]	Using ~1 g of sediment				Using ~0.25 g of sediment			
	HB	IF	HA	MA	HB	HB	HB	HB
Max	27.24	17.54	16.80	41.14	91.97	16.233	137.7	26.1
Min	0.84	0.96	1.24	0.66	8.32	3.90	3.06	25.4
Average (SD)[b]	13.14 (±4.61)	7.38 (±2.54)	17.77 (±7.57)	7.29 (±2.58)				22.23 (±13.43)

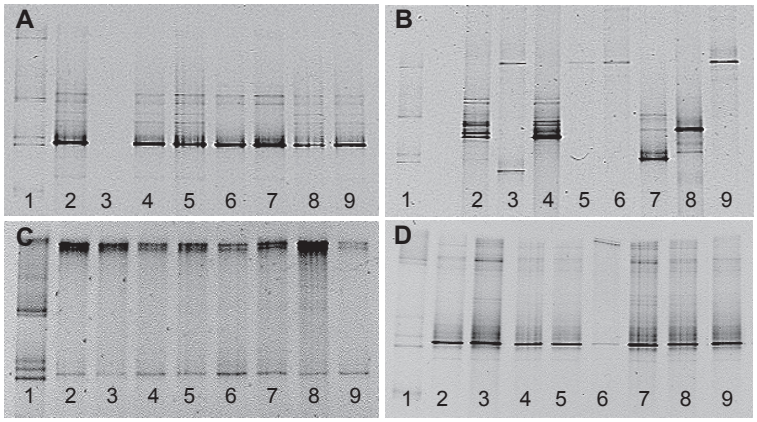
[a] DNA content calculated as ng DNA per g of wet sediment (ng DNA/g sed); shown are maximal and minimal values among 8 individual extractions.
[b] SD is the calculated standard deviation in DNA content amongst 8 (left 4 columns) or 32 individual DNA extractions (right 4 columns).

Horizontal reproducibility of PCR-DGGE in small-scale mesocosms

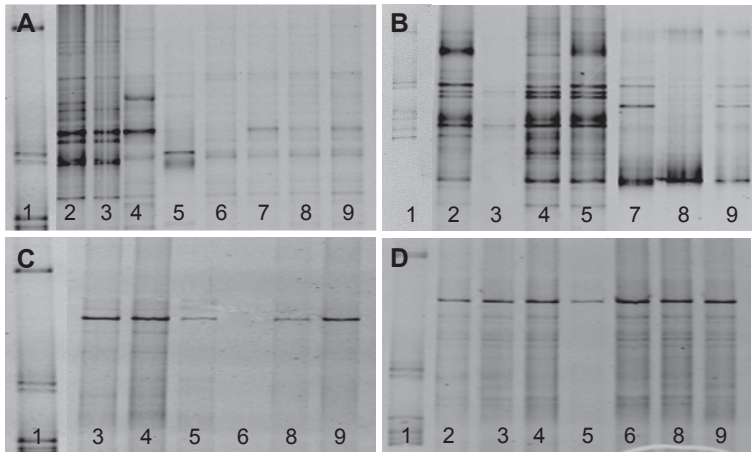
In order to determine the reproducibility of PCR-DGGE fingerprinting on marine sediments, eight small scale mesocosms were sampled randomly, three times, in a horizontal direction, and the fourth sample was a mixture of these three. Following DNA extraction, DNA fragments were amplified with primers specific for the 16S rRNA genes of Archaea, Bacteria and Cyanobacteria, and analysed by DGGE. Figure 1 depicts the results for the cyanobacterial communities and Figure 2 shows the outcome for the Archaeal populations.

As can be seen in Figure 1A-D the fingerprinting of cyanobacteria was successful and reproducible in all cases, although to a lesser degree in sediment IF. Samples taken within one mesocosm were practically identical and the replicate mesocosms gave very similar profiles. The samples from IF showed more variety in patterns, suggesting that the oxygenic phototrophs were not evenly dispersed on the surface of the sediment. For Archaea reproducible DGGE patterns were found for sediments MA and HA (Fig. 2C and D, respectively), yet slightly more heterogeneity was encountered in DGGE profiles of sediments HB and IF (Figure 2A and B, respectively). For these latter two, reproducibility within one mesocosm seemed significant, but somewhat larger differences were found between DGGE profiles of replicate mesocosms.

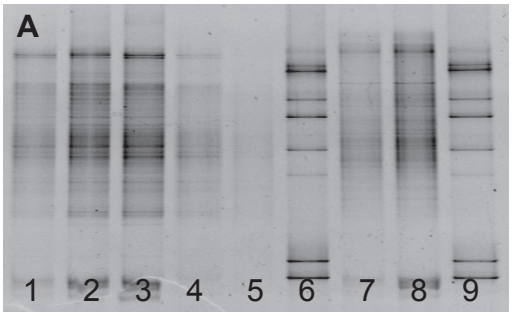
Amplification of DGGE fragments from Bacteria proved to be more problematic and required moderate optimization of the PCR reaction. In most cases, dilution of target DNA and/or addition of BSA, DMSO or extra $MgCl_2$ increased the amplification efficiency, as is shown in the overview of bacterial DGGE profiles in Figure 3. Figure 3A shows reproducible bacterial DGGE profiles of MA (lanes 1-5) and HA (lanes 7-8) sediments. On a different DGGE gel, Figure 3B, MA samples were analysed again in order to check the reproducibility between gels (lanes 1-4). Replicate samples from HB and IF sediment samples are shown in lanes 5-7 and 8-10, respectively. Although differences in band intensities are apparent, the overall pattern within one small-scale mesocosm was considered sufficiently reproducible.



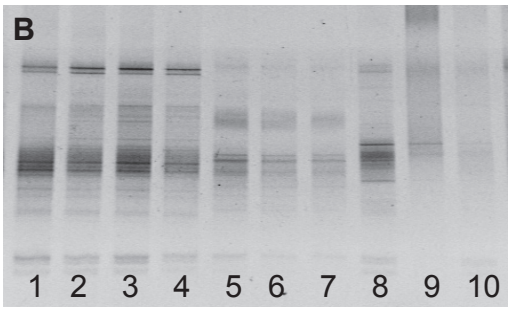
< Figure 1: Reproducibility of cyanobacterial PCR-DGGE profiles in small-scale mesocosms. A: HB sediment, B: IF sediment, C: MA sediment and D: HA sediment. Lane 1: molecular marker; lanes 2-4: horizontal replicates from mesocosm 1, lane 5: mixture of samples 2-4, lanes 6-8: horizontal replicates from mesocosm 2 and lane 9: mixture of samples 6-8 (A-D).



< Figure 2: Reproducibility of archaeal PCR-DGGE profiles in small-scale mesocosms. A: HB sediment, B: IF sediment, C: MA sediment and D: HA sediment. Lane 1: molecular marker; lanes 2-4: horizontal replicates from mesocosm 1, lane 5: mixture of samples 2-4, lanes 6-8: horizontal replicates from mesocosm 2 and lane 9: mixture of samples 6-8 (A-D). If a certain sample (lane) is not represented on the DGGE gel, PCR amplification was not successful.



< Figure 3: Reproducibility of bacterial DGGE profiles in small-scale mesocosms (A and B). A: Bacterial DGGE profiles from MA sediment (lanes 1-5) and HA sediment (lanes 7 and 8). Lanes 6 and 9 are molecular markers. B: Bacterial DGGE profiles from MA sediment (lanes 1-4), HB sediment (lanes 5-7) and IF sediment (lanes 8-10).



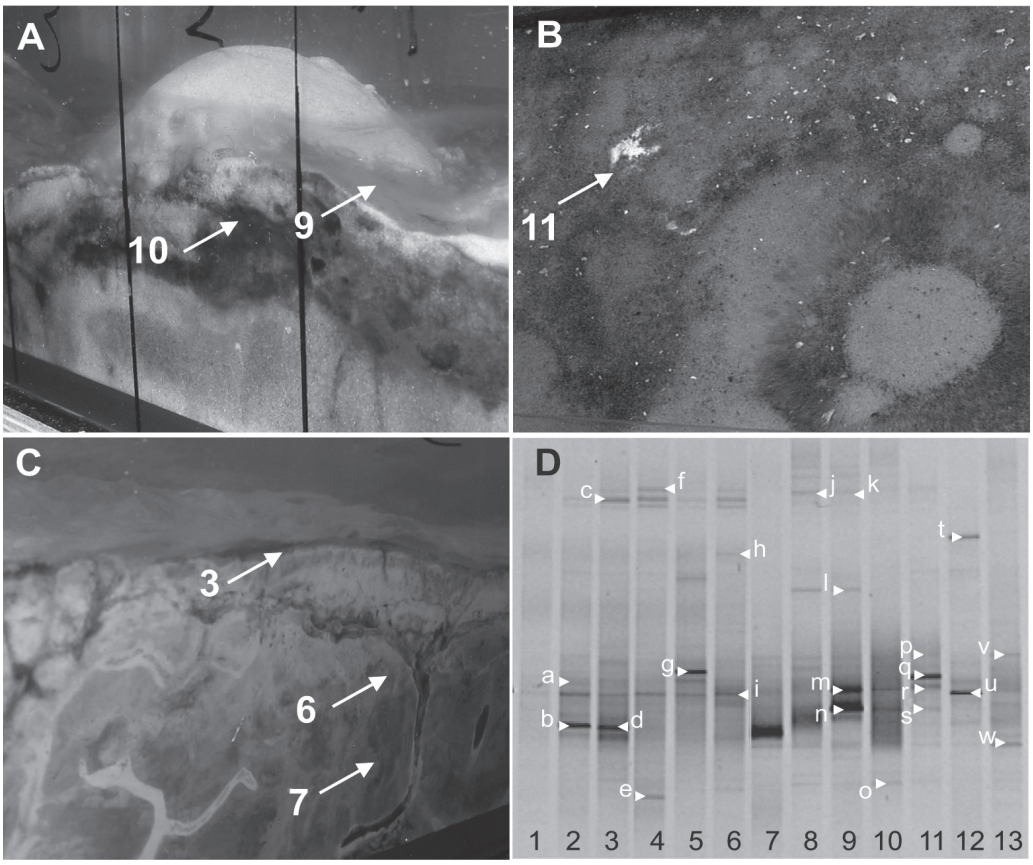
> Figure 4: Structural heterogeneity in large-scale mesocosm sediments as evidenced by visual observations and bacterial DGGE profiles. A: cross-section of IF sediment, B: top view of IF sediment, C: cross-section of HB sediment and D: bacterial DGGE profiles from visually distinct sediment samples. Lane 1 shows DGGE profile for pooled sediments before DNA extraction (HB), lanes 2 and 8 show profiles obtained by pooling samples after DNA extraction (HB and IF, respectively). Lanes 3-7 contain typical samples from HB sediment; 3: phototrophic, 4: iron-oxidizing, 5: sulfur-oxidizing, 6: iron-reducing and 7: sulfate-reducing. Lanes 8-13 contain similar samples for the IF sediment. Some sampling locations are depicted with arrows and lane numbers in the photographs. DGGE bands a to w were excised and sequenced.

Spatial heterogeneity of sediments in large-scale mesocosms

In the large mesocosms, spatial heterogeneity was expected to be greater due to better preservation of sediment structure. In addition, a higher flow rate ensured adequate flushing as well as maintenance of geochemical gradients. Therefore, it was important to obtain some knowledge on the spatial heterogeneity of the "undisturbed" microbial populations, in both the horizontal and vertical direction, before starting contamination experiments.

As can be seen in the photographs in Figure 4A-C, sediments in the large-scale mesocosms displayed patchiness on the surface and clear vertical zonation. A phototrophic biofilm covered large parts of the sandy IF sediment (Fig. 4A) and at the (presumably) oxic-anoxic interface thick iron depositions could be seen. In Figure 4B a patch of bright white material is highlighted at the surface of the IF sediment which might suggest the formation of sulphur. Visual observation of cross-sections of the silty HB sediment indicated the presence of bioturbating worms (Fig. 4C) and iron-deposits lining former burrows.

In an attempt to characterize as many of these potential microbial habitats as possible, five types of locations were sampled per mesocosm. These samples targeted the following general microbial communities (based on colour, structure and depth): phototrophic, sulphur-oxidizing, iron-oxidizing, iron-reducing and sulphate-reducing micro-organisms or methanogens (the latter two samples were taken from ~2 and ~3cm depth, respectively). DNA extraction and amplification of the bacterial DGGE fragments from these samples led to the diverse patterns seen in Figure 4D. Lane 1 shows the PCR DGGE profile resulting from pooling sediments before extraction (samples thereafter used for lanes 3-7), showing a vague smear with no excisable bands. When DNA extracts were pooled, however, DGGE profiles were improved, as is seen in lanes 2 and 8 (containing PCR product of mixed DNA template, i.e. from separate extractions, lanes 3-7 and 9-13, respectively). Sequencing of excised DGGE bands (depicted a to w) and subsequent phylogenetic analysis resulted in the tree displayed in Figure 6 and is presented in detail in the final section of the results.



Vertical distribution of microbial populations in large-scale mesocosms

In submerged sediments with high organic load geochemical gradients are usually steep and transition from oxic to reduced conditions can occur within a few mm's. Benthic microbial communities generally reflect this vertical zonation, as they depend on the various gradients or are responsible for their existence. This section describes the results of DGGE depth profiles from microbial populations, in order to get a detailed picture from these "undisturbed" sediments. With the aim of improving the resolution of sampling, replicate sediment cores were taken and sliced at different intervals.

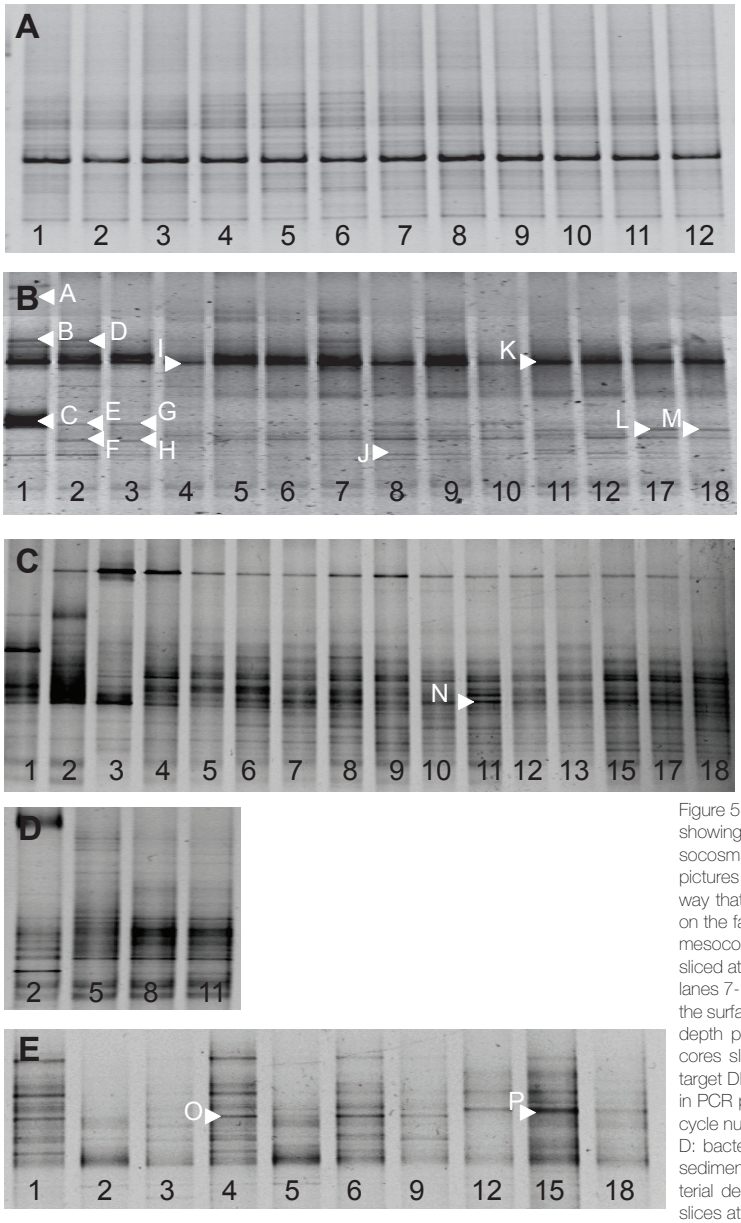


Figure 5: Archaeal and bacterial DGGE profiles showing vertical distribution in large-scale mesocosms at different levels of resolution. In all pictures, the samples are arranged in such a way that the surface sediments are displayed on the far left. A: archaeal depth profiles in HB mesocosm of two replicate sediment cores sliced at 1cm resolution (lanes 1-6: core 1 and lanes 7-12: core 2; samples 1 and 7 represent the surface of the sediment). B and C: bacterial depth profiles in HB mesocosm of sediment cores sliced at 0.5 cm resolution, using 1 µl target DNA and low cycle number (20 repeats in PCR program) (B) or 0.1 µl DNA and higher cycle number (37 repeats in PCR program)(C). D: bacterial depth profiles in IF mesocosm of sediment cores sliced at 0.5 cm and E: bacterial depth profiles in HB mesocosm of filter slices at 0.2 cm resolution.

DGGE profiles from these experiments in the mesocosm with the silty sediment (HB) are shown in Figure 5A-C; sediment slices of 1cm (Archaea) (Fig. 5A) and of 0.5cm (Bacteria) (Fig. 5B-C). DGGE patterns shifted substantially when decreasing the amount of target DNA and increasing the cycle number in PCR amplification (Figure 5C), apparently allowing a wider range of target sequences to be amplified. Overall, these three pictures present a very homogeneous distribution of Archaea and Bacteria in the vertical direction. However, at the 0.5cm resolution, the first two surface samples display different dominant DGGE bands than samples from the lower regions (Fig 5B-C). Figure 5D shows Bacterial DGGE patterns of some sampled depths in the IF sediment. Interestingly, the pattern of the first sample deviates again from the other depths, similar to the HB sediment profiles.

The last sampling technique involved an innovative methodology, as described in the Experimental Procedures section. Basically, cellulose filters were inserted into the sediment, incubated for several hours, carefully removed and subsequently sliced in pieces, which were subsequently used for DNA extraction. This approach allowed DGGE depth profiles at the 2mm-scale, results of which are shown for the bacterial population of sediment HB in Figure 6E. DNA yields of the filter pieces were rather low, but relatively free of inhibitors compared to DNA extracts from sediment samples, allowing higher target concentrations in amplification. Despite optimization some samples still showed poor profiles, but changes in community composition with depth were evident.

Phylogenetic analysis

A wide diversity of Bacteria was identified in the DGGE gel in Figure 4D, with representatives of the *Bacteroidetes*, the α-, δ- and γ-subclasses of the Proteobacteria detected, as well as some chloroplasts and one Gram-positive bacterium. The phototrophic sample of HB sediment (lane 3, Figure 4D) contained members related to *Roseovarius tolerans* (band d, Figure 6), in addition to *Skeletonema*-related sequences (band c); The DGGE profile of the green biofilm on top of IF sediment (lane 9) revealed more members of this clade within the α-Proteobacteria (bands m and n). The sample taken from "sulfur-rich" regions was dominated by a *Sulfitobacter* species in the case of HB sediment (lane 5, band g). In IF sediment (lane 11) two very similar sequences were found (bands q and r), related to an environmental clone from another North Sea study (Eilers et al., 2000).The sequence of DGGE band o represented a member of a deep-branching lineage in the γ-Proteobacteria capable of sulfur-oxidation and was detected in IF sediment between iron-rich deposits at the oxic-anoxic interface (lane 10). Both the HB and IF sediment showed the prevalence of bacteria related to *Shewanella marisflavi* (bands i and u, respectively), when targeting the iron-reducing region of the sediments (lanes 6 and 12). The only sequence related to the δ-subclass of Proteobacteria, was detected in the sulphate-reducing zone from IF sediment (lane 13) and showed highest sequences similarity with *Myxococcales*-like organisms (band w). Different DGGE bands of IF sediment profiles were associated with the *Bacteroidetes* (bands l, p, t and v) detected at all depths in the IF sediment (lanes 8-13).

The DGGE gel represented in Figure 5B, i.e. mesocosm HB, shows succession of microbial populations with depth. The upper sediment layer (lanes 1 and 2) contained sequences related to the *Firmicutes* (bands A, E and G), *Photobacterium* sp. (bands B and D), *Pseudoalteromonas* sp. (band C), *Halomonas* sp. (bands F and H) and *Cytophaga* sp. (bands I and K) (Figure 6). This latter species represented the most dominant DGGE band in Figure 5B at all depths. Solely in deeper layers, i. e. at ~4 and ~8.5cm depth, respectively, a *Shewanella*-like sequence was detected (band J) and species related to *Bdellovibrio* sp. (bands L and M).

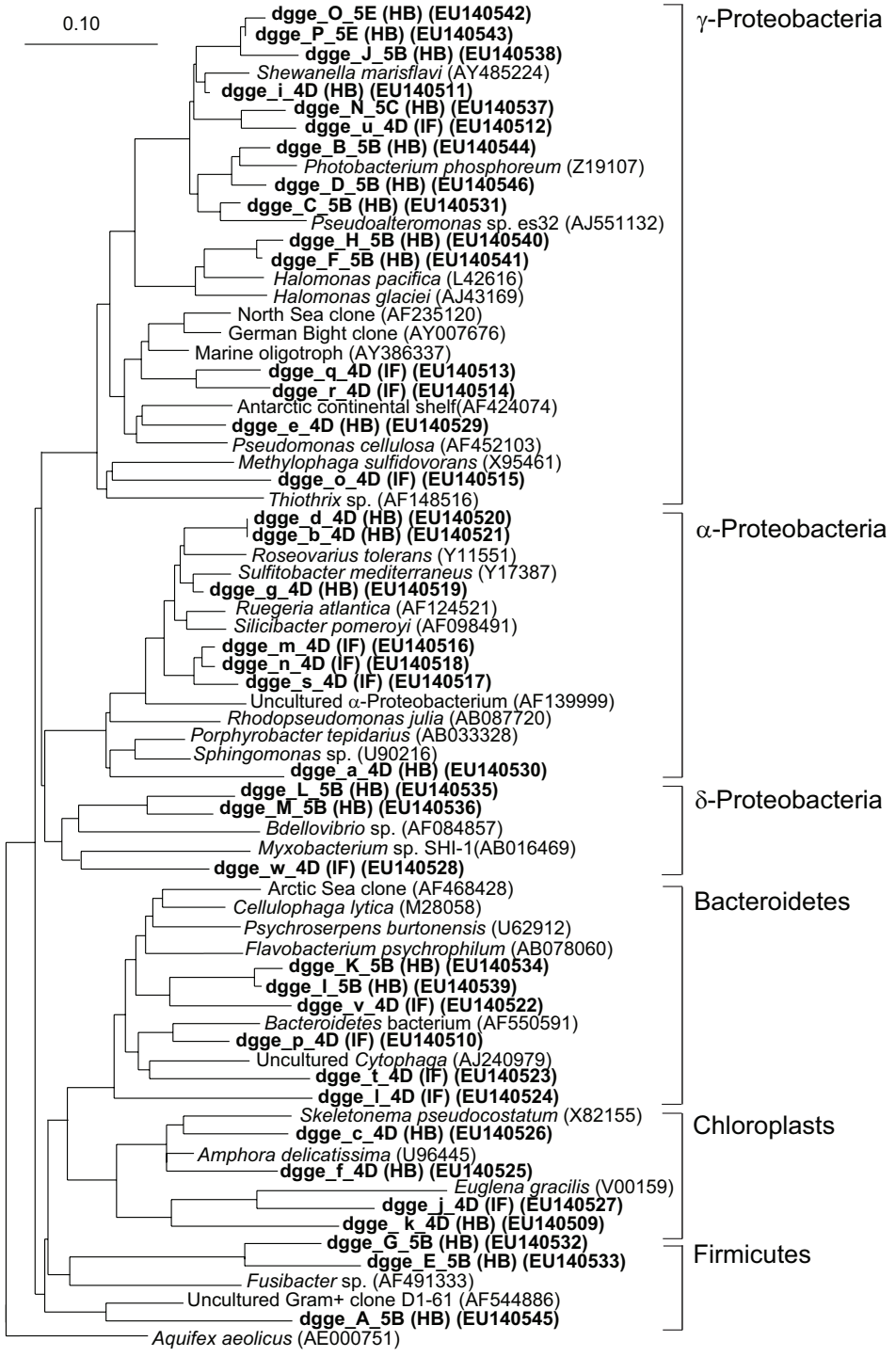


Figure 6: Neigbour joining tree based on 16S rDNA sequences of excised DGGE bands (printed in **bold**), showing the phylogenetic affiliation of community members from the different mesocosms. Origin of sample (e.g. HB or IF) and sequence accession numbers are given between brackets. The tree was rooted with *Aquifex aeolicus* and the scale bar represents 0.10 changes per nucleotide.

Discussion

Different methods to extract genomic DNA from environmental samples as soils and sediments have been tested and published (Steffan et al., 1988; Zhou et al., 1996; Bürgmann et al., 2001). In our study, especially the sandy sediments (IF and MA) repeatedly yielded lower DNA concentrations (Table 2), but this may simply be caused by the fact that microbial biomass was less in these sediments. Zhou and co-authors stated that DNA yield was positively correlated with organic carbon content in soils (1995), confirming our findings of high DNA content in silty sediments rich in organic matter (HA and HB). As has also been stated by Bürgmann and co-authors (2001), it is practically impossible to develop one DNA extraction protocol that will give optimal results for each type of soil or sediment. In order to truly evaluate DNA extraction efficiencies between different sediments, one would have to spike samples with an internal standard before DNA extraction, as described by Petersen and Dahllöf (2005).

Another potential source of bias in microbial ecology is PCR amplification of environmental DNA. Several factors may influence the kinetics of PCR and determine the outcome of amplification, e.g. template concentration, secondary structure of primer and template, G+C content, the number of different templates and the presence of inhibiting substances (Suzuki & Giovannoni, 1996; Nikolausz et al., 2005; Petersen & Dahllöf, 2005). Indeed, changing the amount of template concentration and cycle number had a substantial effect on the observed community composition as detected by DGGE fingerprinting (Figure 5B and C). Mixing of different sediment samples before DNA extraction often had a detrimental effect on the spectrum of DGGE patterns, when compared to the number of bands in the individual samples (Figure 1B, lanes 5 and 9, Figure 2A, lane 5 and Figure 4D, lane 1). By mixing DNA from different extractions before PCR amplification (Figure 4D, lanes 2 and 7) DGGE profiles were generally improved; more bands could be distinguished and some of the DGGE bands were identical to bands from individual samples. However, several dominant bands in individual DGGE profiles could not be found in mixed samples (e.g. DGGE band g from lane 5 was not detected in the profile of lane 2, Figure 4D). Differential or preferential amplification as observed here may be caused by re-annealing of template DNA, which compromises the hybridisation of primers (Suzuki & Giovannoni, 1996). Alternatively, variation in template G+C content or binding affinity of primers, can also differentially impact PCR amplification of mixed templates and therefore result in an incomplete view of the microbial community. Other potential problems are the formation of chimeric or heteroduplex molecules, which can affect the distribution of bands in DGGE profiles, as elaborated on previously (Muyzer & Smalla, 1998). Despite these pitfalls, it can be hypothesised that the degree of preferential amplification is similar across samples, if it is assumed that microbial diversity is comparable in all samples analysed.

Reproducibility of PCR-DGGE within small-scale mesocosms, for Bacteria, Archaea and Cyanobacteria was moderate to good (Figures 1, 2 and 3). This might lie within the line of expectation for silty fine-grained sediments, as these represent relatively impermeable sediments and are generally diffusion-controlled. Contrastingly, due to the high permeability of sandy sediments, coarser grained systems are generally defined as convection controlled. This implies that sediment structure is determined by currents through the sediment, which may result in frequent flushing of one site whereas an adjacent patch is not reached by the flow of water. Indeed, the sandy IF sediment appeared to be heterogeneous to such an extent, that differences in microbial population were sometimes observed within and between mesocosms (Figure 1B, 2B and 3B). However, differences in DGGE profiles were generally small and reproducibility of the applied method was considered sufficient to continue experiments with the larger scale mesocosms. These results seemingly contradict a recent study with small-scale mesocosms, showing little reproducibility in phototrophic community composition between identical mesocosms maintained at different labs (Roeselers et al., 2006). However, community structure in phototrophic biofilms on glass slides is largely determined by light availability, whereas microbial communities in sediment mesocosms are primarily driven by the geochemical equilibrium between solid and liquid phase, which may represent a more constant driving force and hence form a more stable (and "reproducible") microbial community.

Sediments in the large-scale mesocosms showed visual signs of heterogeneity and stratification, particularly in the sandy IF sediment, and these observations were largely confirmed by the variety in bacterial DGGE profiles of selected samples (Figure 4A-D). A number of excised DGGE bands could be linked to ecological functions. For instance, sediment samples with sulfur deposits contained sequences related to known

sulfur-oxidizers from the marine *Roseobacter* group, as *Sulfitobacter* sp. (DGGE band g, Figure 6) (Moran et al., 2003) and samples taken within the metal-reducing regions, indicated the dominating presence of iron-reducing *Shewanella* (DGGE bands i and u) (Nealson & Myers, 1992).

Vertical depth profiles of Archaea and Bacteria in HB sediment showed little variability at 1cm and 0.5cm resolution (Figure 5A-C). Primary changes in microbial populations occurred at around 1cm depth (Figure 5B, 5C), coinciding with the average oxygen decline at approximately 8mm, and therefore possibly indicative of a separation between oxic and anaerobic communities. In the upper sediment layers, typical marine opportunistic γ-Proteobacteria were detected as *Pseudoalteromonas*, *Photobacterium* and *Halomonas* (DGGE bands B, C, D, F and H). In lower regions sequences related to (facultative) anaerobes as *Shewanella* (DGGE band J) and *Bdellovibrio* (DGGE bands L and M) were found. At 2mm resolution, subsequent DGGE patterns show variable profiles (Figure 5E). These results are in accordance with Grundmann's findings (2004), who described high small-scale diversity at millimetre scale in soil systems. In order to establish whether specific microbial groups are associated with a metabolic function at a certain depth, further analysis is needed as, for example, methods based on community 16S rRNA or functional genes.

In conclusion, the application of PCR-DGGE in small-scale mesocosms for bacterial, archaeal and cyanobacterial communities in marine sediments was found to be very reproducible for the silty sediments used in this study (HA and HB) and the sand-silt mixture (MA). The sandy sediment (IF) showed more spatial heterogeneity. In the large-scale mesocosms, patchiness in both sediments (HB and IF) was evident from visual observation (Figure 4A-C) and confirmed by DGGE profiles of selected samples (Figure 4D). Thorough analysis of PCR-DGGE patterns of samples in the horizontal as well as the vertical direction, and phylogenetic identification of individual bands, enabled an initial characterization of the microbial communities in sediments HB and IF, which can serve as a control for the contamination experiments. The fact that sequences related to *Shewanella marisflavi* sp. were detected in several DGGE profiles obtained by different approaches of sediment HB (bands i, J, N, O, P in Figure 6), suggests that i) this micro-organism is a numerically significant member of the Bacterial community and that ii) different sampling strategies and PCR conditions gave comparable results.

Although technology has enabled geochemists to measure nutrients, oxygen, metals, e. a. at micro-scale levels by inserting needle-like sensors in sediments, similar non-destructive yet adequate sampling poses microbiologists for a problem. This study provides a basis for more concise microbiological sampling techniques. While it is generally assumed that microbial communities in sediments are stratified as a consequence of vertical geochemical gradients, this investigation actually documents these transitions. In addition, it was shown here that small changes in microbial populations with depth may be overlooked when using a 1cm sampling resolution. At the 0.5cm resolution, the surface sediments clearly deviated from the lower regions, whereas at 2mm resolution more changes in microbial diversity could be observed.

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Chain of buckets filled with dredged harbour sediment

3 Effects of deposition of heavy metal-polluted harbour mud on microbial diversity and metal-resistance in sandy marine sediments

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Abstract

Deposition of dredged harbour sediments in relatively undisturbed ecosystems is often considered a viable option for confinement of pollutants and possible natural attenuation. This study investigated the effects of deposition of heavy metal–polluted sludge on microbial diversity of sandy sediments, during 12 months mesocosm incubation. Geochemical analyses showed an initial increase in pore water metal concentrations, which subsided after three months of incubation. No influence of the deposited sediment was observed in Denaturing Gradient Gel Electrophoresis (DGGE) profiles of bacterial 16S rRNA genes, while a minor, transient impact on the archaeal community was revealed. Phylogenetic analyses of bacterial 16S rRNA clone libraries showed abundance of members of the *Flavobacteriaceae*, the γ- and α-Proteobacteria, in both the muddy and the sandy sediments. Despite the finding that some groups of clones were shared between the metal-impacted sandy sediment and the harbour control, comparative analyses showed that the two sediments were significantly different in community composition. Consequences of re-deposition of metal-polluted sediment were primarily underlined with cultivation-dependent techniques. Toxicity tests showed that the percentage of Cd- and Cu-tolerant aerobic heterotrophs was highest among isolates from the sandy sediment with metal-polluted mud on top.

Introduction

Different degrees of heavy metal pollution have been observed in coastal areas of North-Western Europe, mostly attributed to industrial discharges, waste disposal streams and atmospheric deposition of exhaust gasses. Stringent environmental legislation has led to a reduction of these metal discharges in the last 15 years (EEA Report, 2003). However, considerable heavy metal pollution of sediments from harbours and marinas have been attributed to the application of antifouling paints on ship hulls (Schiff et al., 2004; Warnken et al., 2004). Some of these harbours have to be dredged frequently for navigational purposes, as is the case for harbours in North-Western Europe from which more than 200 million cubic meters of contaminated sludge is dredged on an annual basis (Bortone et al., 2004). Dredging operations can increase metal mobilization by whirling up fine sediment particles and allowing oxygen to come in contact with previously buried and reduced sediments. The extent of metal release depends on local parameters such as sediment geochemistry, currents, grain size, pH and salinity (Van den Berg et al., 2001). Unquestionably a variety of biological parameters also play a role in metal mobilisation.

When sediment contamination is low, several methods are available to avoid (potential) dispersion or release of toxic metals. Risk assessment studies show that in situ capping (confinement by an inert barrier) or passive natural attenuation offer viable alternatives to removal of sediments by dredging (Wang et al., 2004). However, when metal-contaminated sediments need to be removed for navigational purposes, the most common practice is to simply relocate the dredged material in the same system, with the assumption that this procedure has minimal effect on biotic and abiotic parameters (Bortone et al., 2004), and hence will not lead to release of toxic metals. This study therefore investigated the effect of deposition of dredged metal-contaminated sediment on microbial diversity and metal resistance of microbes in uncontaminated sandy sediment.

Bacterial heavy-metal resistances studies have been carried out mostly on pure cultures (Silver, 1996; Nies, 1999; Miao et al., 2004, Surosz and Palinska, 2004). Recently, some insight has also been gained into the genetic flexibility of mixed microbial populations with respect to metal contamination and widespread occurrence of lateral gene transfer in response to metal toxicity (Sobecky et al., 1998; Cook et al., 2001). While effects on phytoplankton and archaeal communities are described, most studies have focused on bacterial diversity in marine sediments with long-term impact by heavy metals (Gillan et al., 2005; Sorci et al., 1999; Powell et al., 2003). An investigation of biodiversity in several polluted and pristine Antarctic sediments showed substantial statistical variation between and within control groups (Powell, 2003). Furthermore, although Sorci and co-authors observed an increase in biodiversity along with heavy metal contamination (1999), other investigators measured no change (Gillan et al., 2005), or the exact opposite response (Sandaa et al., 1999a). These differences may be explained by adaptation time and/or co-contamination with organic material, but these assumptions remain speculative. Therefore, this study was limited to better controlled conditions. We used (relatively) short-term exposure to metals and employed mesocosms in order to minimize abiotic variances and to enable frequent sampling.

We describe the effects of controlled disturbance ("dredging") and re-deposition of metal-polluted silty sediment on a sandy sediment, by combining diversity assessments of Bacteria, Archaea and cyanobacteria with temporal and spatial profiles of metals. Four mesocosms were subjected to selected treatments mimicking deposition of metal-polluted sediment, micro-scale disturbances (i.e. bio-turbation by Nereis diversicolor) and seasonal variation (i.e. algal bloom by addition of Spirulina sp.). Geochemical analyses of the sediments comprised measurements of nutrients, oxygen and heavy metals throughout the duration of the project (16 months). Microbial diversity studies focused on the first two cm of surface sediments and included 16S rDNA DGGE (denaturing gradient gel electrophoresis) analysis for the domains Bacteria and Archaea, as well as for the phylum cyanobacteria. Bacterial diversity was further explored by construction of 16S rDNA gene clone libraries, and metal-toxicity tests on aerobic heterotrophic isolates.

Experimental procedures

Description of field sites

In June 2003 intact blocks of sediment were collected from a metal-contaminated harbour basin (HB) and a geographically related intertidal flat (IF) on the North Sea coast of Germany. The HB sediment was sampled at 11 m depth with a box-corer and comprised primarily of fine-grained material (78.6% of the grains was smaller than 20 μm). Hydrocarbon contamination was detected in the harbour sediment (poly chlorinated biphenyl: 41-175 mg/kg dry wt and poly aromatic hydrocarbon: 14-18mg/kg dry wt).

The sediment from the intertidal flat comprised mainly of coarser grains (99.5% of the grains ranging from 200 to 600 μm, M. Huettel, personal communication). Salinities in the harbour basin and the intertidal flat were low, 28‰ and 29-30‰ respectively, reflecting the input of freshwater from a river situated 3 km west of the harbour region. The intertidal flat sampling site has been described elsewhere (de Beer et al., 2005; Musat et al., 2006).

Mesocosm design, treatments and sampling

For controlled and time dependent measurements, mesocosms were set-up and run during the duration of the project (16 months, June 03-Oct 04). Four glass aquaria, each 120x30x20 cm, were filled with sediments (36L, or approximately 38,5 kg dry wt of IF sediment and 21.9 kg dry wt of HB sediment), to a height of 10cm. The mesocosms were filled with North Sea water, resulting in a water column of 10cm on top of the sediment (36L). Four mesocosms were established with i) polluted sediment (HBC), ii) homogenized polluted sediment (HBH), iii) sandy sediment (IFC) and iv) sandy sediment with a 3mm layer of polluted sediment deposited on top (IFD).

Water (salinity 27-30‰) was permanently circulated with a metal-free pump at flow rates ranging from 9 and 10.5cm s^{-1}. In order to compensate for evaporation, de-ionized water was added when necessary. The mesocosms were kept at constant temperature (19°C) and illuminated 12 hours per day with fluorescent tubes (T5/Biolight, daylight 80W), at a total irradiance of 40-50μmol photons m^{-2} s^{-1} at the sediment surface. Measurements of pH in the water column of the mesocosms showed little variation over time, i.e. 8.0±0.1 (HBC and HBH) and 7.8±0.1 (IFC and IFD).

Mesocosm HBC contained undisturbed sediment from an industrial harbour basin. Sediment from the same location was homogenized in a cement mixer for 20 minutes under aerobic conditions, before being placed in mesocosm HBH, mimicking mixing processes as might occur during dredging. Mesocosms IFC and IFD contained sediments from an intertidal flat of the German Wadden island Sylt. After an initial stabilization period of three months (June 03-Oct 03), 1L of metal-contaminated sediment was added to mesocosm IFD in October 2003, forming a layer of about 3mm thickness on the sediment surface. In November 2003, bio-turbation was introduced in all sediments by the addition of 100 individuals of the polychaete Nereis diversicolor. In February 2004, a spring algal bloom was mimicked by the addition of organic matter (30g C m^{-2}) in the form of algae (80% dried *Spirulina* sp. (Aldrich) and 20% macroalgae collected from a beach). In September 2004, an additional 50 to 80 individuals of *Nereis diversicolor* and *Arenicola marina* were added to each mesocosm. Survival time of the polychaete worms in mesocosms HBC and HBH varied from 2 to 5 months, whereas in mesocosms IFC and IFD they lasted throughout the project.

Samples for DNA extraction, as well as samples for the culture-dependent experiments, were taken from the top 2cm of surface sediment with sterile, cut-off, syringes (2.5ml). In all cases, samples from three different points in the mesocosms were taken and pooled together, in order to minimize variation due to heterogeneity.

Pore water carbon, nutrients, oxygen and metal analysis

Pore water was extracted by centrifugation of the sediment (sampled with a small corer, 2.6cm diameter, and 1cm thick slices resulting in 5.3ml of sediment) in acid washed Spinex' (Phenomenex) filter units at 2500g for 10min. Samples for nitrate, ammonia, phosphate and silicate were measured as described previously (Hansen and Koroleff, 1999). Sulphate concentrations were measured with an ion chromatograph (Ferdelman et al., 1997). Total carbon (TC) and total organic carbon (TOC) were measured in freeze dried sediment samples with the CNS Analyzer (Fisons Eager 200). For the TOC measurements the samples were acidified with 6N HCl prior to analysis to release inorganic carbon. Oxygen consumption and production rates were determined from the change of the oxygen concentration in the water column. The mesocosms were closed without gas phase and the water column oxygen concentration during light and dark incubation was monitored with oxygen micro-optodes (Microx TX3, Presense). For determination of pore water metal concentrations, were amended with 100µl 12N HCl to prevent oxidation of the metal. The samples were centrifuged and the supernatant was stored at 4°C until analysis on the ICP-MS (Zhang and Davison, 1999). A certified reference material (SLRS-4 from National Research Council of Canada) was measured routinely. The values obtained were within the standard deviation of the certified values. The diffusive flux of dissolved constituents across the sediment-water interface can be calculated according to Fick's first law of diffusion with a modification appropriate for sediments. In this work metal fluxes were calculated for each mesocosm using the concentration gradient of DET (diffusive equilibrium thin gel technique; Zhang and Davison, 1999) metal measurements over a 4mm distance immediately below the sediment surface. Diffusive gels were prepared using acrylamide solution and an agarose derivative cross-linker (DGT Research Ltd), as described by Zhang and co-authors (1995). DET probes were deployed for 56 hours in the sediment. Non-linearity in some concentration gradients could introduce errors in some calculations.

Cultivation of heterotrophic bacteria and metal toxicity assays

Filter sterilized mesocosm water (5ml) was added to pooled sediment samples (~ 6g wet sediment) and shaken rigorously for 10 minutes. Of this solution, 10, 25 and 50ml was used to inoculate agar plates containing a mineral medium (1L contained: 1g NH_4Cl, 0.2g $MgSO_4$.H2O, 0.1g $CaCl_2.2H_2O$, 0.05g $K_2HPO_4.3H_2O$, 27.5g NaCl, 10mmol HEPES, 5mmol acetate, 10mg yeast extract, 1ml of standard trace element solution and 15g agarose, pH 7.5 (Widdel and Pfennig, 1984)). After 24hrs of incubation at 25°C, individual colonies were picked with sterile toothpicks and used as inoculum for new plates, until 30 monoclonal ("pure") cultures per mesocosm were obtained (excluding mesocosm HBH since initial experiments did not indicate any differences with HBC). Due to the presence of isolates with agarolytic traits in collections from IFC and IFD, fewer isolates could be tested on agar diffusion assays, i.e. 21 and 23, respectively.

Isolates were subjected to zone of inhibition assays with filter discs impregnated with either 500nmol of $CuSO_4$ or 150nmol of $CdCl_2$ in duplicate. Previous tests with variable amounts of copper and cadmium on impregnated discs, showed that the applied concentrations resulted in an adequate separation of tolerant and sensitive bacterial species. Plates were incubated at 25°C for 72 hrs, prior to measuring the zone of growth inhibition with a ruler. Bacteria were identified as metal-tolerant when growth occurred within a 5mm zone of the centre of the petridish and as metal-sensitive when zone of growth-inhibition was larger than 12mm.

DNA extraction and 16S rRNA gene amplification

Extraction of genomic DNA was performed on sediment samples (0.25-0.5g of wet sediment) with the Ultra Clean Soil DNA Isolation Kit (MoBIO Laboratories, USA) according to the manufacturer's manual. Genomic DNA was used for amplification of DGGE fragments and the nearly complete bacterial 16S rRNA gene. All PCR reactions were conducted in 50 ml reactions with approximately 50ng target DNA using the Taq PCR Master Mix kit (QIAGEN, Germany), in a Thermocycler (BioMetra, Germany). PCR products were analyzed by gel electrophoresis (1% agarose gel, 30 minutes, 100V), stained with ethidium bromide (0.6mg/ml) and visualized with the BIO-Rad Gel Doc 1000 under UV illumination.

Bacterial DGGE fragments were amplified as described previously with primers 341F-GC and 907R (Schäfer and Muijzer, 2001). DGGE fragments from cyanobacteria and chloroplasts were amplified with primer 359F-GC and an equimolar mixture of the reverse primers 781R(a) and 781R(b), and PCR conditions as described by Nübel et al. (1997). DGGE fragments of Archaea were amplified with primers Parch519F and ARC915R-GC, and PCR conditions as described previously (Coolen et al., 2004).

The nearly complete bacterial 16S rRNA genes were amplified with forward primer 63f-mod and reverse primer 1387r-mod (Marchesi and Weightman, 2000). The PCR reactions were conducted according to the following program: 94°C for 5min (1 cycle), 94°C for 1min, 65°C for 1min, 72°C for 3min (30 cycles) and final extension at 72°C for 10min (3 cycles).

DGGE and MDS analyses

DGGE was performed as described previously (Schäfer and Muijzer, 2001). Approximately 400ng of PCR product was loaded per lane. For the analysis of bacteria and cyanobacteria, gels with denaturant gradients of 20-80% were run for 16h at 100V. The archaeal community was analyzed by running gels with a 20-60% denaturing gradient for 5h at 200V. Gels were incubated for 30min in an ethidium bromide solution (0.5mg/ml) and photographed using the GelDoc UV Transilluminator. In order to assess changes in the microbial communities over time, the DGGE patterns were analyzed with MDS as described previously (Schauer et al., 2000). Digital DGGE images were analyzed with ImageJ 1.36b (Wayne Rasband, National Institutes of Health, USA; http://rsb.info.nih.gov/ij). This program creates a density profile through each lane detecting bands and their intensity. For each DGGE gel, these values were used to create a species-abundance matrix, which was imported into the statistical program Primer-E (version 6, UK Department for Environment, Food and Rural Affairs). Data was standardized by applying a square root transformation, after which a resemblance matrix was created using Bray-Curtis similarity. Multi-dimensional scaling (MDS) configuration plots were generated and these graphs received an overlay of cluster analysis of the same samples (based on similarity, chosen percentages of 50, 60 and 75%).

Clone libraries and screening

Three clone libraries were prepared with near complete 16S rRNA genes amplified from mesocosm HBC (May 2004 and Oct 2004, HBC2 and HBC3, respectively) and from mesocosm IFD (October 2004, IFD3). Initially, the PCR product was purified by gel electrophoresis (2% [w/v] agarose gel), excised and processed with the QIAquick Gel Extraction Kit (QIAGEN, Germany). The PCR product was ligated in the PCR® 2.1-TOPO® vector and used to transform TOP10 chemically competent E. coli cells, according to the manufacturers manual (Invitrogen Life Technologies, the Netherlands). Of the positive clones 38, 101 and 103 clones from HBC2, HBC3 and IFD3 respectively, were subjected to colony PCR of the insert using primers 63f-ext and 1387r-ext (Marchesi and Weightman, 2000). ARDRA screening (amplified ribosomal DNA restriction analysis) with restriction enzyme RsaI was done at 37°C for 2 hours. Restriction products were separated by gel electrophoresis (2% [w/v] agarose gel, 180 minutes, 80V), stained and visualized as described above. The restriction patterns were clustered manually. Of each cluster, 2 to 4 representative clones (except for groups with only one representative) were selected for complete sequencing of the insert. Plasmids of selected clones were purified with the QIAprep Spin Miniprep Kit (QIAGEN, Germany).

Sequencing, phylogenetic analysis and accession numbers

Insert containing plasmids were sequenced with universal primers M13 forward (-20) (5´-GTA AAA CGA CGG CCA G-3´), M13 reverse (5´-CAG GAA ACA GCT ATG AC-3´) and internal primer 907R. Excised, re-amplified and purified DGGE bands were sequenced with the appropriate forward primer, lacking the GC-clamp. All DNA sequencing reactions were carried out on an ABI 3730 sequencer (Applied Biosystems, USA). Partial clonal sequences were combined in the web-based program CAP sequence assembly machine to form a contig (http://bio.ifom-firc.it/ASSEMBLY), of which the remaining primer sites were removed. Modified sequences were compared to sequences stored in GenBank using the BLAST

algorithm (Altschul et al., 1990; www.ncbi.nlm.nih.gov/BLAST) and subsequently imported into the ARB software program (Ludwig et al., 2004; www.arb-home.de). Alignments were corrected manually when necessary. A phylogenetic tree was created with the neighbour joining algorithm. Shorter sequences, such as the DGGE bands, were inserted into this tree one at a time, while applying a filter focusing only on the positions of the shorter fragment. Clonal 16S rRNA sequences were deposited at GenBank under accession numbers DQ334608 to DQ334670 and EF137898 to EF137903. Excised, re-amplified and identified DGGE bands were deposited under accession numbers EF137873 to EF137897.

WebLIBSHUFF analysis and calculation of diversity indexes

A similarity matrix, calculated with Jukes-Cantor correction, was exported from ARB software and used to estimate coverage curves and comparisons between clone libraries with the web based program webLIBSHUFF (J. R. Henriksen, 2004; http://libshuff.mib.uga.edu/) From this similarity index, a species abundance file was created by grouping similarity coefficients higher than 95% (chosen OTU definition). Multiplication with the corresponding frequency of the ARDRA cluster gave rise to species-abundance data. This file was used as input for EstimateS, a web based program that allows for the determination of several non-parametric biodiversity estimators (Version 7.5, R. K. Colwell, http://purl.oclc.org/estimates). Diversity settings included the use of the classical formula for the Chao2 estimator, setting the sample randomization to 1 and putting the upper abundance limit for rare species to 3. Coverage was calculated using the method of Good (Good, 1953) with the equation $C = (1-(n_1/N))*100$, where n_1 is the number of unique clones within a library and N the total number of clones examined. Coverage of clone libraries is a parameter that gives information about how well the retrieved data resembles the original sample, by using statistical information on the frequencies of rare clones. Richness estimators are used to compare biodiversity between different samples and take into account both the number of species and their relative abundance.

Results

Sediment characterization and mesocosm incubation

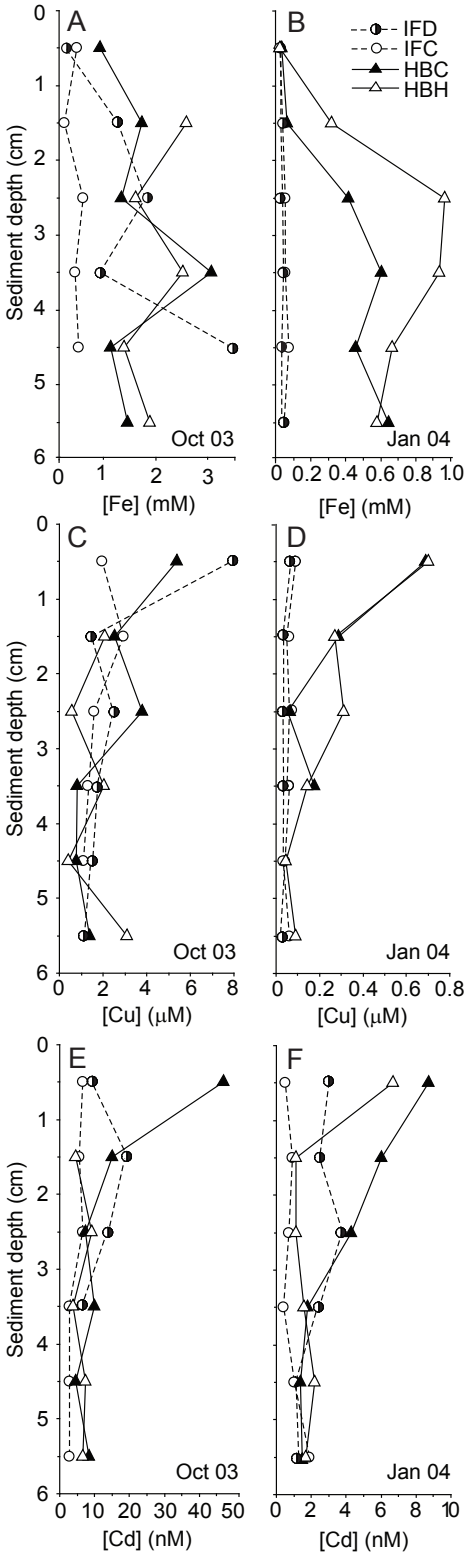
Two marine sediments were used in this study i.e. metal-polluted, fine-grained sediment from a harbour basin (HB) and sandy sediment from a relatively undisturbed intertidal flat (IF) (Table 1). Four mesocosms were established with i) polluted sediment (HBC), ii) homogenized polluted sediment (HBH), iii) sandy sediment (IFC) and iv) sandy sediment with a 3 mm layer of polluted sediment deposited on top (IFD). Description of the four mesocosms, incubation conditions and specific treatments is presented in the Experimental procedures section. Pore water concentrations of ammonia, phosphate and sulphate as well as carbon content were measured during mesocosm incubation in order to investigate the impact of experimental disturbances on abiotic parameters. Oxygen fluxes were measured at the sediment surface interface as indirect indicator of (photosynthetic) microbial activity. Table 1 summarizes geochemical characteristics of the sediments in the mesocosms at different time points; after 3 months in the mesocosms, directly following the deposition of metal-polluted silt on the sandy sediment in mesocosm IFD (Oct 03), again 3 months later, well after the introduction of bioturbation (Jan 04) and after one year of mesocosm incubation (Oct 04). Total carbon (TC) remained more or less stable during incubation in the mesocosms. Homogenisation and re-deposition of polluted muddy sediment (mesocosms HBH and IFD) had minor effects on organic carbon, when compared to the control mesocosms (HBC and IFC, respectively). Ammonia concentrations were elevated in the homogenized polluted sediment (HBH) and remained high after one year.

Table 1: Carbon and nutrients in surface sediments (top 2 cm). HBC: undisturbed harbour sediment; HBH: homogenized harbour sediment; IFC: undisturbed intertidal sediment; IFD: intertidal sediment with a 3 mm layer of HBH deposited on top. Oct 03: samples taken directly after sediment deposition in IFD; Jan 04: samples taken after bioturbation was introduced; Oct 04: samples taken after introduction of organic matter and renewed bioturbation.

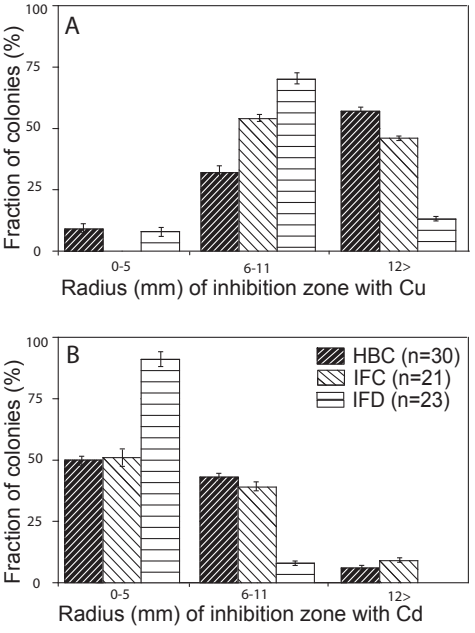
Nutrient:	Time:	Mesocosm:			
		HBC	HBH	IFC	IFD
TC (in mmol C g^{-1} dry wt)	Oct 03	3.5	3.4	0.11	0.11
	Jan 04	3.4	3.4	0.12	0.13
	Oct 04	3.3	3.1	0.27	0.12
TOC (in mmol C g^{-1} dry wt)	Oct 03	1.1	0.74	0.03	0.08
	Jan 04	0.89	0.94	0.04	0.10
	Oct 04	0.86	0.76	0.03	0.05
C/N (total C/total N)	Oct 03	11.3	11.5	6.1	7.4
	Jan 04	10.7	10.6	6.6	7.0
	Oct 04	10.5	10.9	7.2	5.8
NH_4^+ (in μM)	Oct 03	51	110	ND[a]	ND
	Jan 04	55	88	58	31
	Oct 04	43	79	15	19
PO_4^{3-} (in μM)	Oct 03	30	8.3	ND	ND
	Jan 04	15	12	11	9.4
	Oct 04	15	11	13	26
SO_4^{2-} (in mM)	Oct 03	25	23	29	27
	Jan 04	23	20	26	25
	Oct 04	24	28	22	24

[a] ND: not determined

Illumination during daytime resulted in the development of a dense cover of phototrophic biomass in mesocosm IFC and to a lesser extent in IFD. Oxygen penetration depth generally varied between 2 and 8mm in all sediments. Oxygen fluxes were measured regularly at the sediment-water interface in the mesocosms. Oxygen fluxes in the control sandy mesocosm (IFC) remained quite stable for the first three months, around 10mmol O_2 m^{-2} day^{-1} (a positive number indicating transport from sediment to water column), and then steadily increased to 72mmol O_2 m^{-2} day^{-1} in October 2004. In contrast, oxygen production declined rapidly in mesocosm IFD after deposition of silt on the sandy sediment: 22mmol O_2 m^{-2} day^{-1} in September 2003, to 4mmol O_2 m^{-2} day^{-1} in November 2003, and dropping to zero in January 2004. In mesocosms HBC and HBH oxygen production was stable in time, but data indicated lower oxygen production rates, i.e. 7mmol and 5mmol O_2 m^{-2} day^{-1}, respectively.



< Figure 1: Pore water iron (A-B), copper (C-D) and cadmium concentrations (E-F) in the mesocosms during incubation. Symbols shown in 1B are valid for all graphs. Note that the scales of the x-axes of all right hand panels are a factor 3-10 lower. Timing as described in legend Table 1.



v Figure 2: Results from toxicity tests on collections of aerobic heterotrophs for copper (A) and cadmium (B). Symbols shown are valid for both graphs. Metal-toxicity was estimated by measuring zones of growth-inhibition on agar plates amended with filter discs impregnated with copper or cadmium. Error bars represent standard deviation between two replicate experiments.

Metal analysis

Spatial and temporal concentration profiles of iron, copper and cadmium in the mesocosm sediments were measured in order to estimate the extent of metal-release or -immobilization during controlled disturbances. In Figure 1 pore water concentrations are summarized of iron (Fig. 1A-B), copper (Fig. 1C-D) and cadmium (Fig. 1E-F) in the sediments directly after deposition of metal-polluted sediment in IFD (Oct 03) and after 6 months of mesocosm incubation with bioturbation (Jan 04). Please note that the units of the x-axis in the right hand panels are a factor 3-10 lower than the left hand panels. From the first pore water profiles (Fig. 1A-B), it can be seen that iron dominated metal geochemistry in HB sediments and was detected at all depths of the sediment column. The sandy IF sediments had iron concentrations approximately 3 to 5-fold lower, with the exception of the Oct 03 IFD sample, taken directly after metal-polluted mud deposition, showing considerable iron concentrations below 3cm depth. Regarding copper and cadmium, pore water concentrations were elevated in sediment IFD in Oct 03, (Fig. 1C, 1E), while after 3 months, levels were back to the control values (Fig. 1D, 1F, respectively). Both copper and cadmium profiles show maximal concentrations at, or just below, the sediment surface.

Micro-scale pore water metal analysis at the sediment-water interface measured with DET permitted the estimation of metal fluxes as presented in Table 2. Initially, substantial metal fluxes could be measured (i.e. from sediment into water column) in mesocosms HBC and IFD. After three months of bio-turbation most metal fluxes had subsided, except for copper which was relatively high at the end of the project. The homogenized sediment in mesocosm HBH initially exhibited little or no metal fluxes, but metal transport developed after three months and after that declined.

Table 2: Estimated metal fluxes across the sediment water interface for iron, copper and cadmium during mesocosm incubation. Timing as described in legend of Table 1.

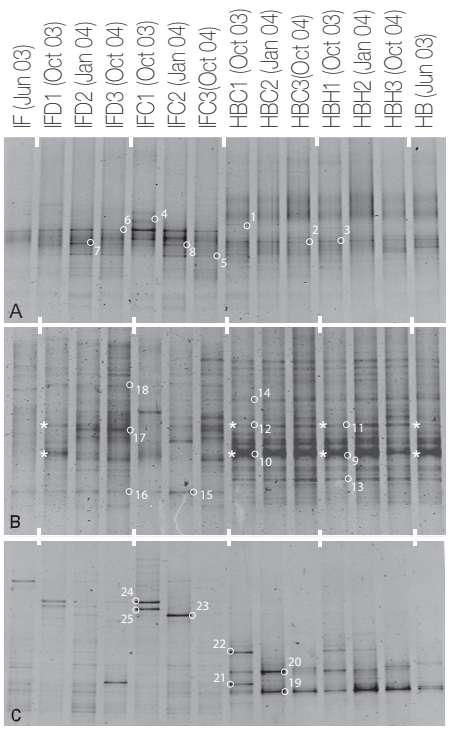
Metal flux[a]:	Time:	Mesocosm:			
		HBC	HBH	IFC	IFD
Fe flux (μmol m^{-2} day^{-1})	Oct 03	980	20	ND[b]	2300
	Jan 04	90	1600	-4	-20
	Oct 04	-28	90	-21	44
Cd flux (nmol m^{-2} day^{-1})	Oct 03	0	-5	ND	160
	Jan 04	0	-4	13	-3
	Oct 04	-4	3	0	0
Cu flux (nmol m^{-2} day^{-1})	Oct 03	ND	ND	ND	ND
	Jan 04	ND	ND	ND	ND
	Oct 04	470	460	-8	98

[a] Positive flux indicates transport from sediment to water column

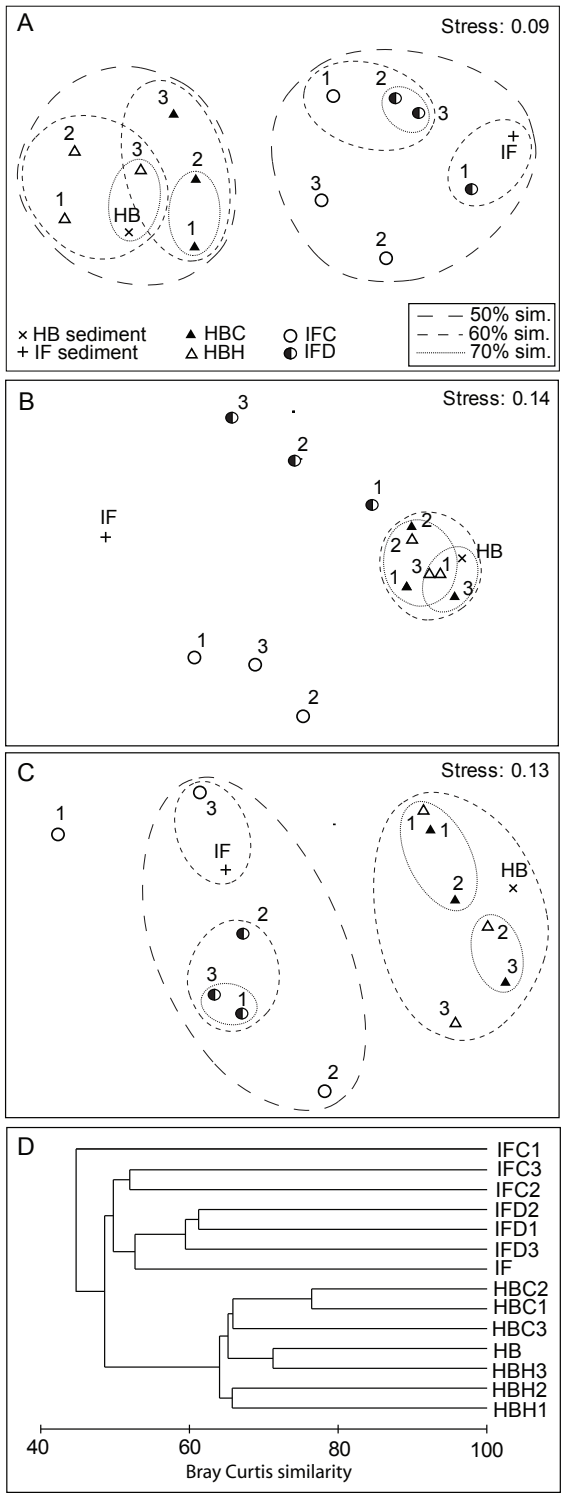
[b] ND: not determined

Metal toxicity tests with aerobic heterotrophic isolates

In order to establish whether the deposition of metal-polluted sediment would increase the number of metal-resistant bacteria, heterotrophic isolates from mesocosms HBC, IFC and IFD were subjected to metal toxicity tests in October 2004. Figure 2 shows the results of these toxicity assays on collections of 30, 21 and 23 isolates originating from HBC, IFC and IFD, respectively. In the case of copper toxicity (Fig. 2A), it was observed that only a modest fraction of isolates from mesocosms HBC and IFD showed resistance, i.e. a zone smaller than 5mm (9 and 8%, respectively, of all isolates in each collection). For mesocosms IFC and IFD the highest fraction of bacteria exhibited inhibition zones between 6 and 11mm (54 and 70%), whereas the majority of isolates from mesocosm HBC (57%) actually turned out to be rather sensitive to copper. In addition, the number of copper-sensitive bacteria in IFC was significantly lower than in IFD.



^ Figure 3: DGGE profiles of bacterial (A), archaeal (B) and cyanobacterial (C) communities at different time intervals in the mesocosms during incubation. Legend above DGGE gel 3A is valid for all graphs. Besides the previously described sampling points (Oct 03, Jan 04 and Oct 04), the outermost left lane shows DGGE profiles of the original intertidal flat sediment as sampled in June 03 (labelled IF) and the lane to the far right shows the original profiles for the harbour sediment (labelled HB). Open circles with numbers depict excised identified bands. DGGE bands marked with asterisks are discussed in the main text.



> Figure 4: Non-metric MDS analyses of DGGE profiles of Bacteria (A), Archaea (B) and Cyanobacteria (C), cluster analysis of all 3 groups combined (D). Symbols shown in the top graph are valid for all graphs. MDS analyses received an overlay of Bray-Curtis resemblance, resulting in clustering according to 50, 60 or 70% similarity.

The second graph (Fig. 2B) shows similar tests with cadmium as toxic agent. The majority of isolates from each mesocosm (HBC: 50%, IFC: 51% and IFD: 91%) showed a zone smaller than 5mm, i.e. relatively resistant against Cd. The second category (6-11mm) comprised most of the other isolates (HBC: 43%, IFC: 39% and IFD: 8%). Bacteria sensitive to cadmium (zones bigger than 12mm) comprised 6 and 9% of the isolate collections from HBC and IFC, respectively, and were not detected at all in the isolate collection of mesocosm IFD. The number of cadmium-tolerant bacteria in IFD sediment was higher than in IFC.

DGGE profiles of sediment samples and statistical analyses

To monitor major changes in the microbial communities during mesocosm incubation, DGGE profiles were constructed of the original sediments (HB and IF) and mesocosm samples taken at three different time points. Figure 3 shows the DGGE results for Bacteria (Fig. 3A), Archaea (Fig. 3B) and cyanobacteria/ chloroplasts (Fig.3). The average number of bands observed per DGGE profile and the total number of bands detected per DGGE gel were for Bacteria 11.7/ 164 (Fig 3A), for Archaea 14.6/ 205 (3B) and for cyanobacteria 12.1/ 170 (3C). As visual interpretation of DGGE patterns appeared to be rather subjective, a statistical tool was used to estimate profile similarity (Schauer et al., 2000). Results of non-metric multidimensional scaling analyses (MDS) are shown in Figure 4 for Bacteria (Fig. 4A), Archaea (Fig. 4B) and cyanobacteria (Fig. 4C). Figure 4D shows the results of cluster analysis of the sediment samples, combining information of 3 above mentioned groups.

The bacterial community profile showed many faint bands. Effects on community structure seemed minimal; the DGGE band patterns did not change much, even after transition from the sampling site to the mesocosms in the lab (lanes IF, IFD1 and IFC1 for the sandy sediment and lanes HB, HBC1 and HBH1 for the harbour sediment). For statistical analysis of DGGE profiles, the stress value of the analysis was lowest for the bacterial community (0.09), i.e. the statistical significance was highest. This graph (Fig. 4A) shows that there was a tight relationship between all samples from the harbour sediments (HB, HBC1-HBH3). Subsequent cluster analysis revealed that the 50% similarity criterion also applied to the collection of sandy sediments (IF, IFC1-IFD3).

Archaeal DGGE profiles showed a very stable community (Fig. 3B) in the case of the harbour sediments (HB, HBC1-HBH3). More variation in the number and positions of bands was found in samples form the sandy sediments. IFD1 and IFC1 showed little similarity and the most dominant two bands in IFD1 were at the same apparent height as major bands in HB, HBC and HBH (denoted with an asterisk). IFC3 and IFD3 showed similar profiles again after 6 months incubation. MDS analyses (Fig. 4B) confirmed these observations as a strong clustering (within 60%) is seen for the harbour sediments (HB, HBC1-HBH3) whereas no significant clustering was found between the IF samples. However, sample IFD1, taken directly after deposition of metal-polluted mud, was closer to the HB samples, whereas samples IFD2 and IFD3 indicated a trend towards IF.

Figure 3C depicts DGGE analysis of cyanobacterial fragments and revealed major differences in the phototrophic communities of the sandy and the harbour sediment. The latter showed profiles of modest diversity (lanes HB, HBC1-HBH3) with almost identical succession patterns. A closer look at the profiles in lanes IF and IFC1-IFD3, revealed a vast quantity of bands and also showed comparable successive changes in the two sandy sediments (IFC1, IFC2, IFD1 and IFD2). MDS analysis of the cyanobacterial gel (Fig. 4C) yielded a clear separation between HB versus IF. Samples from mesocosm IFC showed most variation, especially IFC1 and IFC2. The last graph combining 3 DGGE profiles (Fig. 4D) depicts IFD1-3 and the original sandy sediment (IF) on the same branch, despite the deposition of harbour mud. The samples from the sandy control sediment (IFC) form an outgroup, while all HB profiles were very similar and cluster together.

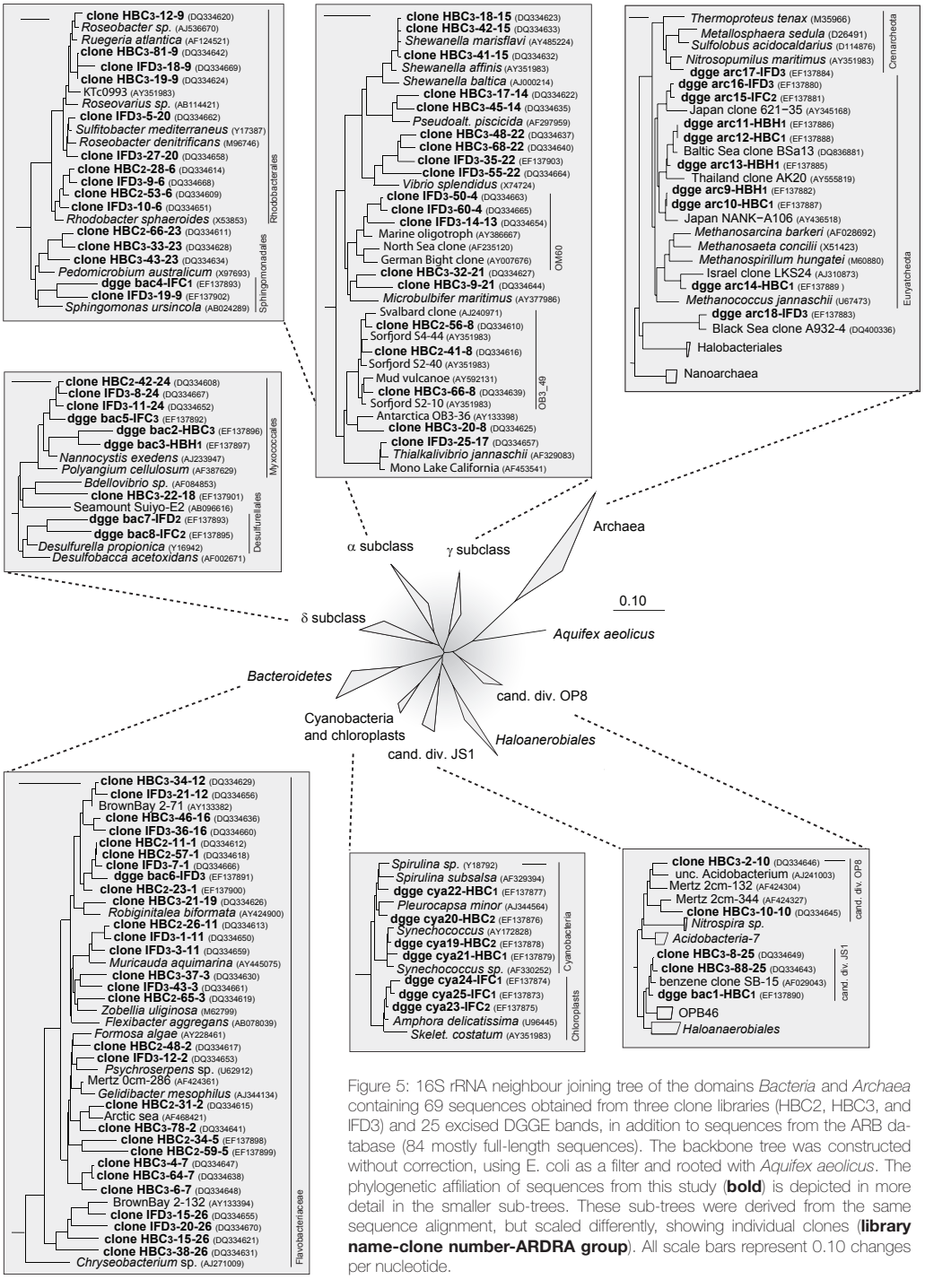


Figure 5: 16S rRNA neighbour joining tree of the domains *Bacteria* and *Archaea* containing 69 sequences obtained from three clone libraries (HBC2, HBC3, and IFD3) and 25 excised DGGE bands, in addition to sequences from the ARB database (84 mostly full-length sequences). The backbone tree was constructed without correction, using E. coli as a filter and rooted with *Aquifex aeolicus*. The phylogenetic affiliation of sequences from this study (**bold**) is depicted in more detail in the smaller sub-trees. These sub-trees were derived from the same sequence alignment, but scaled differently, showing individual clones (**library name-clone number-ARDRA group**). All scale bars represent 0.10 changes per nucleotide.

Phylogenetic analysis of DGGE bands

Most DGGE fragments that were sequenced belonged to the δ-Proteobacteria (bands 2, 3, 5, 7 and 8). Other bands were identified as members of the α-Proteobacteria (band 4), the *Bacteroidetes* (band 6), and a possible member of candidate division OPB46, associated to the *Haloanaerobiales* (band 1). These sequences and all other DGGE bands are represented in the phylogenetic tree in Figure 5. The main tree in the centre shows different lineages present in the sediment samples, while the smaller trees depict a detailed view of phylogenetic diversity. All retrieved sequences from the Archaea were closely related (94-99% similarity) to other environmental sequences deposited at GenBank. One of the most intense bands in IFD3 (i.e. band 17) belonged to the *Crenarcheota*, and was related to the recently isolated ammonia-oxidizing *Nitrosopumilus maritimus* (AY351983). All other bands originated from sequences within the *Euryarchaeota* with closely related to clones from various marine sediments (bands 9-16 and band 18).

Cyanobacteria were found as major representatives of the phototrophic community in harbour sediments, as dominant bands were related to *Synechococcus* sp. (bands 19 and 21), *Spirulina subsalsa* (band 22) and *Pleurocapsa minor* (band 20). In contrast, DGGE analysis of the sandy mesocosms (IFC and IFD) showed the prevalence of mostly diatoms, with all three sequenced bands closely related (95-97% similarity) to chloroplast rRNA of *Amphora delicatissima*.

Screening of bacterial clone libraries and comparative analyses

In order to facilitate a more detailed analysis of bacterial diversity and to verify whether any overlap existed between bacterial communities in the different mesocosms, possibly indicating carry-over of bacteria as a consequence of sediment deposition, a more robust technique than DGGE was required. Therefore, clone libraries were made of nearly complete 16S rRNA genes amplified with target DNA extracted from mesocosm HBC (HBC2 from May 2004 and HBC3 from October 2004) and from mesocosm IFD (IFD3 from October 2004). As a screening method, 38 (HBC2), 101 (HBC3) and 101 (IFD3) positive clones were subjected to restriction analysis. Clustering of all clonal restriction patterns resulted in 26 ARDRA groups, 10 of which were detected in HBC2, 18 in HBC3 and 15 in IFD3. Representative clones of each ARDRA group, 69 in total, were selected for complete sequencing of the small subunit ribosomal gene. These sequences were also included in Figure 5. From the sequences and their assigned ARDRA clusters, it can be derived that in most cases sequence similarity within ARDRA groups varied from 90-99%, though few clusters did not meet this criterion (i.e. groups 5, 10 and 26).

About one third of each library consisted of sequences that were most closely related to uncultured micro-organisms according to similarity analysis with sequences stored in GenBank. The majority of sequences were affiliated to the Bacteroidetes (Fig. 5), i.e. 71% of all clones in HBC2, 47% in HBC3 and 49% in IFD3. Second most abundant were sequences related to the Proteobacteria, in particular to the g- and the a-subclasses (11% and 13% for HBC2, 21% and 17% for HBC3 and 19% and 28% for IFD3, respectively). The δ-subdivision of the Proteobacteria made up only a small fraction of the bacterial community in each library, namely 5% for HBC2, 1% for HBC3 and 5% for IFD3. Remaining 16S rRNA sequences (HBC3) were distantly affiliated to the phyla *Haloanaerobiales* and the *Acidobacteria*, clustering with sequences in candidate divisions JS1 and OP8, representing 9% and 6%, respectively.

Since differences in sample size (number of clones analyzed with ARDRA) and sequencing effort (ratio of sequenced clones versus sample size) existed between libraries, different approaches were combined to estimate and compare diversity. WebLIBSHUFF analysis (Singleton et al., 2001) was selected for library comparison, since it is independent of a strict species definition, but rather computes similarity across all possible cut-off values. Two libraries are considered significantly different when $p<0.05$, as was the case in the comparison of HBC3 to IFD3 ($p=0.001$). Notably, the p-value for the reverse comparisons was much higher ($p=0.374$), indicating that sample IFD3 did not contain many species that were not present in sample HBC3. Analysis showed that HBC2 was very similar to HBC3 ($p=0.783$). To further investigate differences in bacterial diversity between the different sites, richness estimators (Shannon (H') and Chao2) and coverage (C) were calculated, as summarized in Table 3. As uniqueness criterion, a 16S rRNA gene similarity of <95% was applied. Sequences of three clusters in the Bacteroidetes (see Fig. 5), i.e. ARDRA groups 3, 2 and 26, did not meet this criterion and all their members were therefore counted as individuals.

Table 3: Diversity indices and coverage as calculated from clone library data.

Library	# clones	# ARDRA groups (unique)[a]	Coverage (%)[bc]	Shannon (H')[c]	Chao2 (95% CI)[cd]
HBC_2	38	10 (1)	82.2	2.41	75 (36-182)
HBC_3	101	18 (8)	86.8	2.88	248 (137-470)
IFD_3	101	15 (4)	84.3	2.84	203 (111-396)

[a] Total number of ARDRA groups detected in clone library and number of unique ARDRA groups between brackets
[b] Coverage was calculated with the equation of Good (1953)
[c] Coverage, Shannon index and Chao2 estimator were calculated using uniqueness definition of <95% 16S rRNA gene similarity
[d] Between brackets the 95% confidence intervals are listed.

3

Discussion

Metal distribution in mesocosm sediments

The pore water metal contents in the HB sediments were higher than in the IF sediments (Fig. 1), but similar to maximum concentrations observed in other sites throughout the North Sea and river sediments (BSH report, 2002), with the exception of copper. However, pore water copper concentrations detected in mesocosm HBC in January 2004 were at least 50-fold higher, and 10- to 20-fold elevated, when compared to values in other North Sea sediments. This extreme copper pollution is primarily connected to the fact that the harbour basin is located in the vicinity of an industrial wharf (Schiff et al., 2004; Warnken et al., 2004).

Profiles of pore water copper and cadmium concentrations (Fig. 1C-F), peak at the surface or just below. High surface concentrations were probably caused by the microbial oxidation of metal-contaminated organics at the sediment surface (Tankere-Muller et al., 2007). Since oxygen penetration depth varied between different time points, release of copper and cadmium in the subsurface (below 1cm) could also be caused by the anaerobic reduction of heavy metal-containing iron(hydr)oxides (Markwiese and Colberg, 2000). After three months of bio-turbation with Nereis diversicolor iron, copper and cadmium pore water concentrations were lowered approximately 10-fold at all sediment depths, due to mixing by the polychaete worms and subsequent chemical oxidation, adsorption or precipitation.

Perception of diversity

Clone libraries of 16S rRNA genes and DGGE analyses have been widely used to investigate microbial communities of different habitats, but these approaches suffer from specific limitations that have been elaborated on previously (von Wintzingerode et al., 1997; LaMontagne et al., 2002). Since this study does not aim to describe diversity in general, but focuses on specific changes in microbial diversity, the use of identical tools on different mesocosms minimizes variance in methodological biases. It is commonly accepted that only dominant populations (i.e. constituting more than 0.1-1% relative abundance) are detectable in DGGE profiles of complex microbial communities (Muijzer et al., 1993), whereas the detection limit of clone libraries depends solely on the number of clones that are analyzed. Contrastingly, the chance of obtaining a numerically dominant bacterium in isolation depends on how well incubations conditions resemble the bacterial micro-environment. The concentration of carbon source in the culture medium (5mM) was high compared to TOC values in both the sandy and the muddy sediments, and may have triggered opportunistic bacteria. The results obtained by application of two molecular techniques and primer sets for the determination of Bacterial diversity, suggested agreement between the methods, as members of the δ-Proteobacteria, the *Bacteroidetes* and candidate division JS1 were detected independently in both cases. The fact that community composition was not reflected by the collection of DGGE bands in the phylogenetic trees (Fig. 5) can partially be explained by the fact that not all bands within one profile could be identified. The high number of

DGGE bands related to members of the δ-Proteobacteria is in contrast with the low abundance of this group according to clone library results (around 5%) and could indicate primer preferences.

Changes in archaeal and cyanobacterial communities

The archaeal communities in mesocosms HBC and HBD showed a very stable composition during incubation time, whereas DGGE profiles from IFD and IFC were more variable (Fig. 3B and 4B). Sandaa and coworkers (1999b) detected a decrease in Archaeal abundance and a shift in community composition in soils amended with heavy metals. In this study, the latter effect was only initially observed (IFD1) and similarity between DGGE profiles (Fig. 3B and 4B) increased again at the end of the experiment.

Cyanobacterial DGGE profiles (Fig. 3C) showed successive changes in the composition of the photosynthetic community in mesocosms IFC and IFD. These changes may be due to bio-turbation, grazing pressure, or the shift from natural to artificial light conditions (daylight is 20 times stronger than the lamps used). The DGGE profiles were dominated by closely related strains of the diatom *Amphora delicatissima.* Contrastingly, DGGE profiles of mesocosms HBC and HBH revealed a simple and stable community structure consisting of *Synechococcus* sp. and *Pleurocapsa* sp. Lower flow rates during mesocosm incubation may have favoured growth of the rather fragile diatoms in the sandy sediments (Stal, 2003). Phytoplankton species in general are sensitive to copper and other metals due to impairment of the photosynthetic electron transport system (Miao et al., 2005; Surosz and Palinska, 2004). While no attempts were made in this study to quantify the phototrophic biomass, oxygen flux measurements at the sediment-water interface in mesocosm IFD confirmed this sensitivity by showing a steep decline in oxygen production immediately after addition of metal-polluted sludge.

Changes in bacterial communities

Changes in bacterial diversity as a result of the deposition of metal-polluted sediment were not readily observed in the DGGE profiles (Fig. 3A). Comparative analysis between clone libraries HBC3 and IFD3 showed several groups of highly similar, but not identical clones. These “shared” clusters were most closely related to *Ruegeria atlantica, Rhodobacter sphaeroides, Vibrio splendidus* and a group related to environmental clone BrownBay 2-71, within the *Flavobacteriaceae* (Fig. 5). Particularly this latter group showed high 16S rRNA gene similarities between clones from IFD3 and HBC3 (ARDRA group 12: 99% similarity and group 16: 98%). Interestingly, clone BrownBay 2-71 actually originated from a heavy metal-polluted Antarctic sediment (Powell et al., 2003). Statistical comparisons between the clone libraries showed that the bacterial community in sediment HBC3 was significantly different from IFD3 ($p=0.001$). Diversity estimators (Table 3) indicated HBC3 as having the highest species richness, though differences with IFD3 were very small. A similar trend was observed in Antarctic sediments when comparing clone libraries of polluted and pristine sites (Powell et al., 2003). This high diversity may be related to the fact that metal-polluted environments usually also contain many other types of contaminants, such as petroleum hydrocarbons or chlorinated compounds, in addition to increased concentrations of organics and nutrients. Therefore, in an environment poor in organic carbon and nutrients, the selective pressure presumably exerted by heavy metals, may be overshadowed by a diversity increase, due to expanding metabolic possibilities.

Effects of sediment homogenisation and re-deposition

Homogenisation of metal-polluted sediment (HBH) was expected to cause a temporary decrease of metal concentrations in pore waters, due to trapping of metal-ions by previously buried sulphides, or due to adsorption to freshly formed iron oxides (Markwiese and Colberg, 2000). In this study, only small, localized effects were observed for copper and cadmium after sediment homogenisation (Fig 1). Concomitantly, a small fraction of organic carbon was oxidized and ammonia was released (Table 1). A study on the environmental effects of dredging activities in the Pongol estuary, Singapore, (Nayar et al., 2007) also showed elevated levels of ammonia and depletion of organic carbon during and after sediment removal. The effects of homogenisation on metal concentrations observed in this study were in accordance with earlier findings (van den Berg et al., 2000). No important changes were observed in the microbial communities of HB sediments

after sediment homogenisation and incubation (Fig. 3 and 4), indicating that similar micro-organisms were present throughout the sediment column and that the dominant consortium was not influenced by small-scale disturbances. This observation was in sharp contrast with the mesocosms with sandy sediments, which overall showed more heterogeneity and temporal fluctuations.

Deposition of a 3mm layer of metal-polluted sediment on a sandy sediment (IFD) caused a substantial increase in pore water copper concentrations of surface sediments (4-fold compared to IFC and 1.5-fold compared to HBC), and led to significant metal fluxes from sediment to water column (Figure 1, Table 1 and 2). All these effects were of a transient nature and had mostly disappeared after one year of mesocosm incubation. Elevated pore water metal concentrations as a result of re-deposition of polluted sediment were also described in other studies (Leipe et al., 2005). However, the degree of impact depends on the volume of deposited material and its contamination level, as Chen and co-authors (2003) only observed an increase in copper levels after deposition of a 5cm layer of polluted sediment, while no effects could be detected underneath a 1cm layer.

Toxicity tests with isolates underlined the effects of the deposition of metal-polluted sediment by showing a substantial increase in copper- and cadmium-tolerant aerobic heterotrophs from mesocosm IFD when compared to IFC. The fraction of cadmium-tolerant bacteria in IFD was even higher than observed for isolates of mesocosm HBC (Fig. 2). In addition, the collection bacteria from IFD contained the lowest percentage of copper- and cadmium-sensitive bacteria. Elevated levels of metal-tolerant Bacteria in soils and sediments after exposure to heavy metals have been described previously (Diaz-Ravina and Baath, 1996; Ramaiah and De, 2003, Rasmussen et al., 1998), but not in combination with detailed metal analyses indicating such localized and short-term metal exposure.

In conclusion, this study provides convincing evidence for a prolonged modification of the indigenous bacterial community caused by transient exposure to copper and cadmium. It seems unlikely that this adaptation concerns numerically dominant micro-organisms, as large community shifts were not observed in DGGE profiles, except, initially for the archaeal population. It remains to be determined whether the increase in metal-resistance in mesocosm IFD is due to the proliferation of metal-tolerant bacteria, originating from the deposited metal-polluted sediment, or whether horizontal gene transfer of metal-resistance genes may have played a role.

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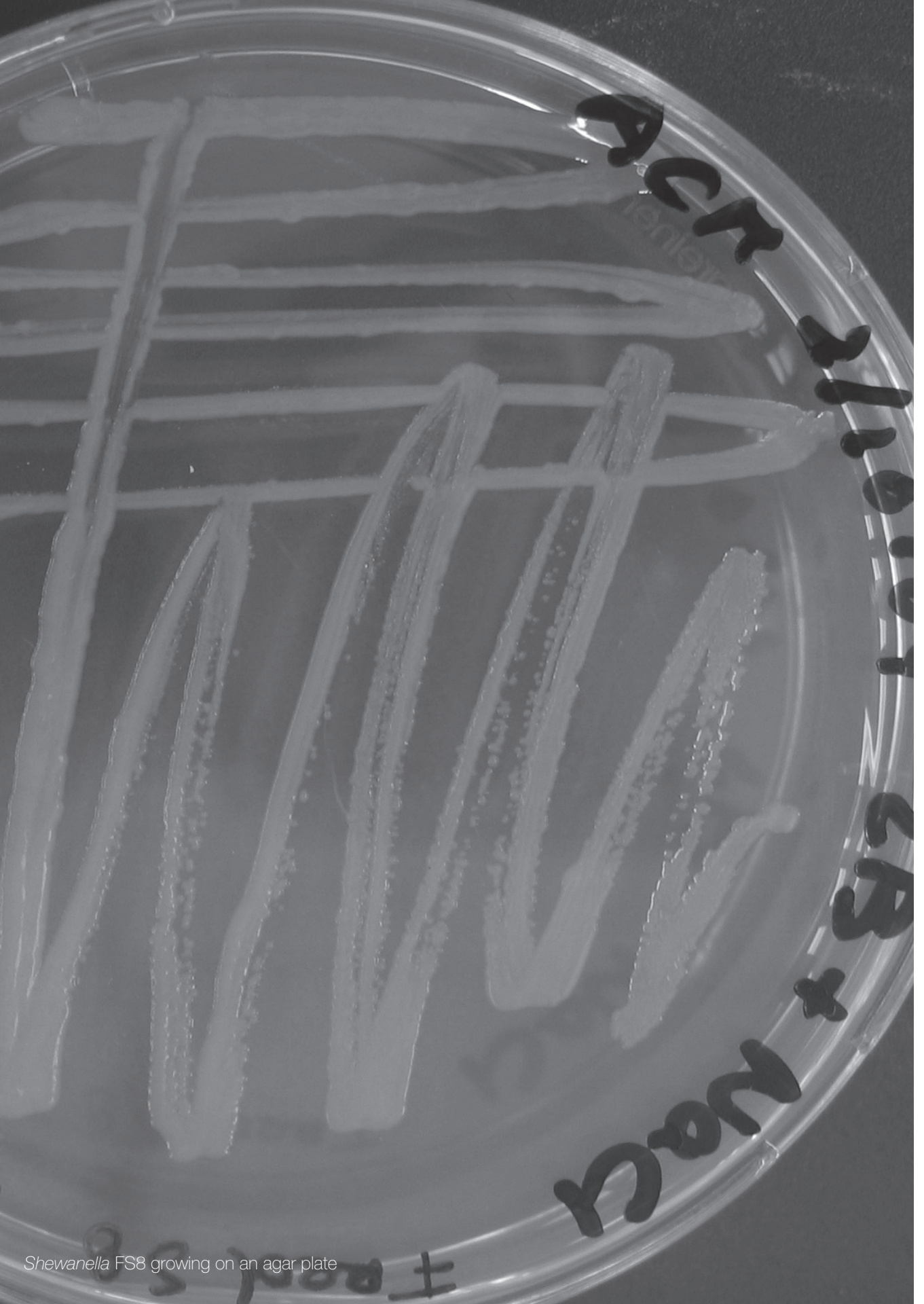

Shewanella FS8 growing on an agar plate

4 Characterisation of heavy metal resistance of Fe(III)- and Mn(IV)-reducing *Shewanella* isolates from marine sediments

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Abstract

This study focuses on heavy metal resistance of marine, benthic Fe(III)- and Mn(IV)-reducing bacteria and their potential to mobilize heavy metals from sedimentary phases, as hydrous ferric oxides (HFO) and Mn(IV)-oxides (δ-MnO_2). One isolate was obtained from enrichments of metal-polluted sediment with δ-MnO_2 (strain MB4, 99% similarity to *S. marisflavi*), and two strains were isolated from enrichments on HFO (strain FB18 and FS8, 98 and 97% 16S rRNA gene similarity to *Shewanella colwelliana*). Toxicity tests under aerobic conditions showed that the latter two ceased growth at 150μM Cu, but strain MB4 and reference strain *S. oneidensis* MR1 were more tolerant to copper; growth with 150μM Cu reached 56-58±0.1% of maximal optical density, OD_{max}, in control cultures. Similar experiments conducted under anaerobic conditions with fumarate indicated no significant change in copper tolerance in strain MB4 (66±3% OD_{max} at 150μM). Biphasic experiments with δ-MnO_2-reduction followed by use of fumarate, furthermore indicated that the presence of manganese oxides decreased bio-availability of copper through sorption processes, thereby alleviating the toxicity of copper to strain MB4 to some extent. Scanning electron microscopic images showed the initial amorphous Mn(IV)-oxides and newly formed, highly crystalline, lemon-shaped particles making up the precipitate that remained after microbial reduction. Concomitant electron dispersive x-ray spectrometry confirmed presence of copper in the initial sample, yet detected no copper in the precipitate after microbial reduction, indicating that the Mn(IV)-reducing *Shewanella* strain MB4 immobilized copper adsorbed to δ-MnO_2.

Introduction

Metals such as cadmium, copper, cobalt and zinc are regarded as serious pollutants of aquatic ecosystems due to their general toxicity and ability to be incorporated into food chains. Underlying sediments are often an important reservoir for heavy metals (Lewis and Landing, 1992; Nasr et al., 2006). In marine systems, where sulfate concentrations are relatively high and the activity of sulfate-reducing bacteria results in sulfide production, a considerable fraction of heavy metals is immobilized as metal-sulfides under anaerobic conditions. Metal sulfides have been shown to be major controlling factors of heavy metal bioavailability in a number of sediments (Cardellicchio et al., 2006; Naylor et al., 2006; Larner et al., 2007).

Other sedimentary phases however, e.g. clay, organic matter and metal-oxides, also may interact significantly with heavy metals. For example, distribution of copper was positively correlated with organic matter content and with the silty, fine fraction in certain sediments (Morillo et al., 2004; Nasr et al., 2006). A metal-polluted, silty harbor basin sediment showed sharply defined maxima of copper and cadmium at 2mm below the sediment water interface, consistent with their release from organic matter as it is oxidized (Tankere-Muller et al., 2007). The general capacity of iron and manganese oxides to adsorb a wide variety of heavy metals is well-known (Takematsu, 1979; Tebo et al., 2004; Abdallah et al., 2007). The addition of iron oxides to remove heavy metals is even part of common practice in certain commercial applications as waste water treatment systems (US patent 6666972, 2003). Taking into account these various potential reservoirs for heavy metals in marine sediments, the question arises whether specific microbial populations, such as iron(III)- or manganese(IV)-reducing bacteria, play an important role in heavy metal mobilization. Potential applications for heavy metal-resistant metal-reducing bacteria, for instance in bioremediation, are mentioned in the literature (Nealson & Saffarini, 1994; Lloyd, 2003; Ruggiero et al., 2005), yet factors governing metal-reduction and associated heavy metal release are only starting to be revealed (Markwiese & Colberg, 2000; Campbell et al., 2006).

Among dissimilatory metal-reducing bacteria, *Shewanella* spp. are perhaps the most widely studied, as well as frequently isolated from aquatic habitats (Ziemke et al., 1998; Venkateswaran et al., 1999; Ivanova et al., 2003). Members of this genus are Gram-negative, facultatively anaerobic, rod-shaped organisms belonging to the γ-subclass of the Proteobacteria. Different *Shewanella* type-strains have been studied elaborately because of their abilities to reduce a variety of metals, among which Mn(IV) and Fe(III), but also Co(III), As(V), Cr(VI), Tc(VII) and U(VI) (Nealson & Myers, 1992; Guha et al., 2003; Saltikov et al., 2003). Generally, few studies concerning metal-resistance compare toxicity under different conditions (Outten et al., 2001), despite the fact that bio-availability (and toxicity) are affected by heavy metal speciation. The speciation of a metal is described as the distribution of the total metal concentration over all its possible chemical forms (Plette et al., 1996), and is influenced by redox potential, pH, available ligands, etc. (Glasby and Schulz, 1999).

The versatile *Shewanella* sp. represent interesting study objects. It was shown that chromate tolerance and reduction rates of *S. oneidensis* MR1 were higher during aerobic growth compared to anaerobic conditions (Middleton et al., 2003; Viamajala et al., 2004). This increase in toxicity may be explained by the fact that sulfate ABC transporters are expressed during anaerobic growth, enabling the structurally similar chromate to enter the cytoplasm. Alternatively, chromate may specifically inhibit anaerobic respiratory chain components or reduced Cr(III) is retained within the cell (Viamajala et al., 2004). Campbell and co-authors showed simultaneous Fe(III)-and As(V)-reduction by *Shewanella* sp. ANA-3, and improved iron-reduction rates when As(III) was sorbed to hydrous ferric oxide (HFO), possibly due to an increase in surface area or crystallinity (2006). With regard to the influence of manganese-oxides on heavy metal toxicity, column experiments with *S. alga* indicated delayed chromate-reduction in presence of pyrolusite (δ-MnO_2) (Guha et al., 2003) and decreased U(VI)-reduction rates in batch experiments with *S. putrefaciens* CN23 and δ-MnO_2 (Liu et al., 2002). These negative effects of δ-MnO_2 on metal-toxicity were ascribed to abiotic re-oxidation of Cr(III) and U(IV) by the manganese-oxides.

The free ion activity model assumes that dissolved metals in the form of "free" cations (e.g. Cu^{2+}, Zn^{2+}) are bio-available, whereas (in)organic metal complexes are not directly available for biological uptake (Worms et al., 2006). Accordingly, we hypothesized and tested whether iron-and manganese-oxides limit heavy metal availability by sorption processes and therewith lower heavy metal toxicity to Fe(III)- or Mn(IV)-reduc-

ing bacteria. Conversely, during microbial reduction of heavy metal-contaminated Fe(III) or Mn(IV) oxides, available heavy metal concentrations are expected to rise and cause toxicity. The present study characterises heavy metal toxicity resistance of three *Shewanella*-like bacteria that were isolated from metal-polluted and pristine marine sediments and provides comparable data for type-strain *Shewanella oneidensis* MR1. Toxicity tests in aerobic conditions were conducted with cobalt, zinc, cadmium and copper. Copper resistance was further investigated under anaerobic conditions, by supplying fumarate, hydrous ferric oxide (HFO) or manganese-oxide (δ-MnO_2) as terminal electron acceptor. Finally, the most copper-resistant isolate was challenged to reduce manganese oxides, pre-equilibrated in copper solutions with concentrations below and above the maximal adsorption capacity of δ-MnO_2 for copper.

Experimental procedures

Enrichment, isolation and cultivation of iron- and manganese reducing bacteria

Anaerobic sediment samples (2-5cm depth) were taken from sediment mesocosms and added to filter-sterilized sea water in anaerobic glass flasks. A homogeneous mixture of sediment bacteria was prepared by vigorous mixing and forcing the sediment slurry through a series of needles with decreasing diameters, and used to inoculate Fe(III)- or Mn(IV)-reducing enrichments. Serial dilution in anaerobic flasks with minimal marine medium resulted in isolation of three strains. This medium contained per litre (g): 1 NH_4Cl, 0.2 $MgSO_4.H_2O$, 0.1 $CaCl_2.2H_2O$, 0.05 $K_2HPO_4.3H_2O$, 27.5 NaCl and 2.38 HEPES. pH was adjusted to 7.5 with NaOH and the medium was sterilised by autoclaving. After cooling, 1ml of trace element and vitamin stock solution (Widdel and Pfennig, 1984) was added. Sterilized glass flasks were filled with 20 ml of medium, iron- or manganese-oxides were added to a final concentration of about ~20mM and the flasks were closed with butyl rubber stoppers. The gas-phase was flushed with filter-sterile argon gas. Subsequently, from an anaerobic stock solution lactate (20mM) and iron(II) (20µM) were aseptically added. The inoculation volume was about 1% of the total volume. Flasks were incubated at room temperature in the dark. Growth was monitored by microscopy, i.e. counting, and metal reduction was visually determined by a change of colour (and volume) of the metal-oxide; the orange brown HFO turned black upon microbial reduction and the brown black manganese oxide was almost completely removed, except for a beige white precipitate remaining.

Preparation of iron and manganese oxides and detection of Fe(II) and Mn(II)

Hydrous ferric oxide (HFO) and amorphous manganese oxides (δ-MnO_2 or vernadite) were prepared as described previously (Nealson & Scott, 2006), with the difference that the metal oxides were not freeze dried before use in microbial cultures. Oxides were washed at least 6 times and progress in removal of salts was monitored by measuring pH and conductivity. Microbial metal-reduction experiments and heavy metal adsorption tests were conducted with freshly prepared oxides, to avoid structural changes and ageing during storage as wet solution. Metal sorption experiments were done by incubating known amounts of oxide in different copper concentrations for two days. The maximal amount of copper sorbed to manganese oxides was estimated by incubating different amounts of oxides in copper solutions and subsequently measuring the residual copper (C_{eq}, in mg Cu/l) with flame-AAS technique. Plotting C_{eq} versus Q_{eq}, i.e. copper adsorbed per amount of oxide (in mg Cu/g oxide) leads to an asymptotic curve, approaching the value 0.14mg Cu/g oxide or 4.5mg Cu/g dry wt of oxide. Dry weight determination of metal-oxides was determined after drying in an oven at 105°C.

Mn(II) was determined with a modified version of Goto's protocol (1962): to 1ml of filtred sample, 50ml of formaldoxim reagens (1g hydroxylamine in 35ml of 40% (w/v) ammonia with 0.5ml formaldehyde), 150ml 1M NaOH, 50ml 0.1M EDTA and 100ml hydroxylamine (10% w/v) were added and absorbance was measured 450nm. Fe(II) was measured with the ferrozine method, as described by (Viollier et al., 2000).

Aerobic metal toxicity tests

S. oneidensis MR1 (Myers & Nealson, 1988) was kindly provided by K. Nealson from the University of Southern California, LA, USA. All cultures were kept as glycerol stocks at -80°C. Each test was carried out with cultures that had not been previously exposed to heavy metals (unless otherwise noted). Before the actual toxicity tests, each culture was transferred three times to new medium after approximately 8 hrs of growth. The growth medium used in these experiments consisted of 10-fold diluted LB broth (per liter: 1g tryptone, 0.5g yeast extract and 5g NaCl) with extra salt added for the three marine strains (27.5g total). Tests were also done in 60%-LB, 40%-LB and minimal medium (described above) with 10mM lactate supplemented and 0.01%(v/v) yeast extract.

Aerobic toxicity tests were carried out in 96-well plates (clear polystyrene plates with U-shaped bottom, total volume of 200µl, Matrix technologies). Each well was filled with 99µl of medium, and all wells (except the outer columns and rows) were inoculated with 1µl of cell suspension. The plates were closed with lids and incubated in a Tecan plate-reader, allowing for temperature control (at 20°C), automated shaking and optical density measurements (at 660nm) during incubation. All tests were done at least in triplicate, including no-metal and no-cells controls. Average standard deviation between replicates was less than 0.5%. $BaCl_2$ was initially used as a density standard and together with plate weighing before and after 72 hrs incubation, the total loss of medium due to evaporation was calculated to be lower than 3% at room temperature (2.9µl/well/ 3days at 20°C, but 5.9µl/well/ 3days at 30°C). Metals were added from (acidified) stock solutions: $CuSO_4.5H_2O$ (50mM), $CoCl_2.6H_2O$ (10mM), $CdSO_4.H_2O$ (10mM) and $ZnSO.7H_2O$ (10mM). In addition, larger scale tests in glass flasks containing 10, 25 and 50 ml of culture were conducted in the same medium and incubated under similar conditions (in duplo). Results of metal toxicity tests throughout this paper are presented by the maximal OD (OD_{max}) measured during growth in presence of heavy metals (within 48 hrs), compared to the maximal optical density measured in control cultures without metal, and expressed as a percentage.

Anaerobic metal toxicity tests

For anaerobic metal-toxicity tests, cells were pre-grown aerobically as described above. Anaerobic flasks containing the minimal marine medium also used for initial isolation were prepared with lactate as carbon source and either fumarate (20mM), iron(hydr)oxide (20mM) or manganese oxide (20mM) as final electron acceptor. For *S. oneidensis* the salt content was lowered to 5g/l. Growth with fumarate was very limited, unless some yeast extract was added (0.01v/v%). Copper was added to the anaerobic flasks with a syringe; typically, larger inocula were applied (2.5%), compared to aerobic tests. Colony forming units (CFU) were determined by stepwise (10x) dilution of culture samples in fresh medium and streaking 50ml of at least 3 dilutions in duplicate on plates (containing 10-fold diluted LB and 15g/l agar).

DNA extraction, amplification and DGGE analysis

Extraction of genomic DNA from sediment samples and bacterial cultures was conducted with the Ultra Clean Soil DNA Isolation Kit (MoBIO Laboratories, USA) according to the manufacturer's manual. Quality check and quantification of total genomic DNA was done with the Nanodrop ND 1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). All PCR reactions were conducted in 50 ml reactions with approximately 50ng target DNA using the Taq PCR Master Mix kit (QIAGEN, Germany), in a Thermocycler (BioMetra, Germany). PCR products were analyzed by gel electrophoresis (1% [w/v] agarose gel, 30minutes, 100V), stained with ethidium bromide and visualized with the BIO-Rad Gel Doc 1000 under UV illumination. Bacterial DGGE fragments were amplified with primers 341F-GC and 907R and analysed as described previously (Schäfer and Muyzer, 2001); per lane 400ng of PCR product was loaded. The nearly complete 16S rRNA genes of isolates were amplified with forward primer 63f-mod and reverse primer 1387r-mod (Marchesi and Weightman, 2000). The PCR reactions were conducted according to the following program: 94°C for 5min (1 cycle), 94°C for 1min, 65°C for 1min, 72°C for 3min (30 cycles) and final extension at 72°C for 10min (3 cycles). Gyrase B genes were amplified with *Shewanella* specific primers as described by Venkateswaran and co-authors (1999).

Phylogenetic analysis

In this study we analyzed the complete 16S rDNA and the *gyrase B* genes of 3 bacterial isolates. In addition, partial 16S rDNA genes were retrieved from DGGE profiles amplified with DNA of benthic microbial communities. The appropriate gene fragments were amplified, excised from gel (re-amplified in case of DGGE) and further purified. The PCR products were sequenced with the appropriate forward primers, lacking the GC-clamp. All DNA sequencing reactions were carried out on an ABI 3730 sequencer (Applied Biosystems, USA). Sequences were compared to sequences stored in GenBank using the BLAST algorithm (Altschul et al., 1990; www.ncbi.nlm.nih.gov/BLAST) and subsequently imported into the ARB software program (Ludwig et al., 2004; www.arb-home.de). Alignments were corrected manually when necessary. A phylogenetic tree was constructed using close relatives from the ARB database and imported sequences from GenBank. Sequences were deposited at GenBank under accession numbers EU147244-EU147246 (16SrRNA genes) and EU147247-EU147249 (gyrase B genes).

Analytical techniques and microscopy

Optical density of cultures was measured with a spectrophotometer at 600nm. Copper concentration was analysed with the flame-AAS technique, on a PerkinElmer AAnalyst 200, callibrated between 0-2mg Cu/l (J. Padmos, Applied Sciences, Technische Universiteit Delft, the Netherlands). Concentration of acetate in 0.2µm-filtered culture samples was determined with High Pressure Liquid Chromatography (HPLC), using an Aminex HPX-87H column from Bio Rad (T=60ºC) coupled to an UV and a RI detector, with phosphoric acid 0.01M used as eluent. Microscopic slides were analyzed with a Zeiss Axioplan 2 epifluorescence microscope and images were acquired with Leica FW4000 software. Samples were visualized with phase contrast settings or incubated with DAPI (4', 6'-diamidino-2-phenylindole) and accordingly treated. Metal oxides and reduced products were analyzed with a Philips Scanning Electron Microscope (SEM), with 2000-2500 magnification and concomitant Electron Dispersive X-ray (EDX) analysis (A. Thijssen, Civil engineering, Delft University of Technology, the Netherlands). Element ratios mentioned in the text represent mean values of at least 3 individual measurements.

Results

Characterisation of iron- and manganese-reducing isolates

Sediment samples from a metal-polluted harbour basin and an intertidal flat were used in this study as inoculum for enrichment cultures selecting iron- and manganese-reducing bacteria. After growth had been confirmed by microscopy and Fe(II)- or Mn(II)-formation, serial dilution resulted in the isolation of three strains: MB4 (enriched on Mn(IV)), FB18 (enriched on Fe(III)) (both from the harbour basin) and FS8 (from the intertidal flat, enriched on Fe(III)). Although the Mn(IV)-reducing enrichment of the intertidal flat sediment also showed growth, no pure culture was obtained. Figure 1A shows PCR-DGGE analysis of the obtained isolates, designated FB18, MB4 and FS8 (lanes 1,2 and 5, respectively) and of the bacterial communities in the original sediments used as inocula (lane 3: harbour basin and lane 4: intertidal flat). Comparative sequence analysis of the complete 16S rRNA genes of the three isolates and sequences from GenBank resulted in the phylogenetic tree shown in Figure 1B. All three isolates were affiliated to the genus *Shewanella*. The strains isolated with Fe(III) as electron acceptor, FB18 and FS8, were closely related to each other (98% gene similarity) and to *S. colwelliana* (98% and 97%, respectively), while strain MB4, isolated with manganese oxide, was most closely related to *S. marisflavi* (99% gene similarity). To further establish the taxonomic position of the isolates, sequences of *gyrase B* genes of these isolates were compared to those from other *Shewanella* sequences (downloaded from GenBank, www.cnr.tigr.org or www.genome.jgi-psf.org). Results (not shown) confirmed the above mentioned relationships.

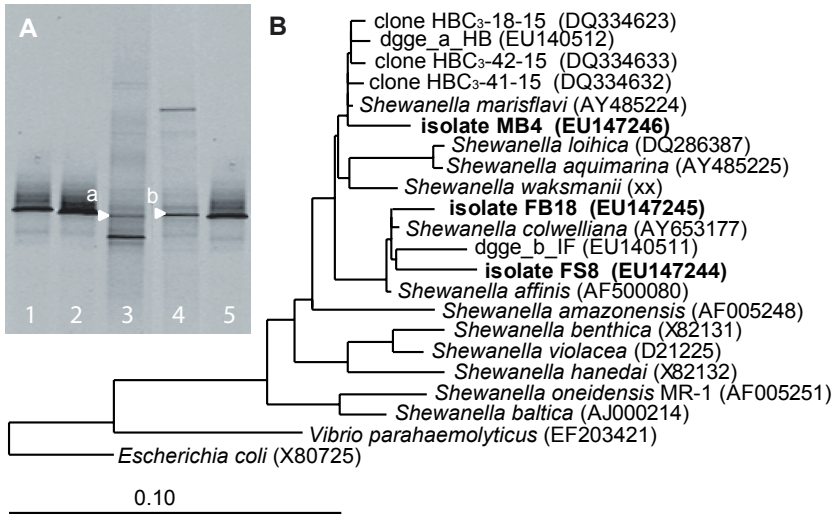
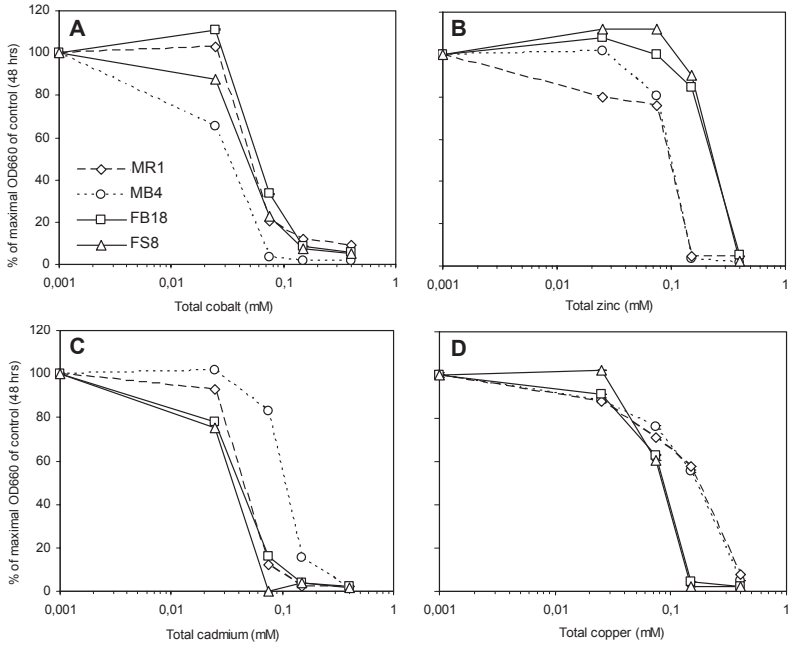


Figure 1A: Picture showing DGGE bands of metal-reducing isolates FB18 (lane 1), MB4 (lane 2) and FS8 (lane 5) in addition to profiles of the original inocula used for isolation from the harbour basin sediment (lane 3) and intertidal flat sediment (lane 4). DGGE bands a and b were excised and sequenced. B: neighbour joining tree based on 16S rRNA sequences showing the phylogenetic affiliation of the three metal-reducing isolates (**bold**) and DGGE bands a and b within the *Shewanellaceae*. Scale bar represent 0.10 changes per nucleotide.

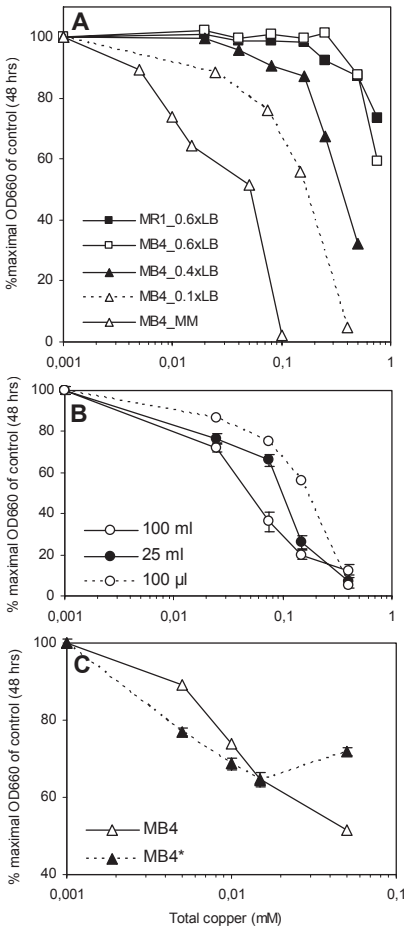
Heavy metal toxicity tests in aerobic conditions

Toxicity tests for four heavy metals were carried out with the type strain *Shewanella oneidensis* MR1 and the three marine *Shewanella* isolates. Optical density (OD, at 660nm) of cultures growing in presence of varying heavy metal concentrations was monitored during 48 hrs. By expressing the maximal OD obtained from metal-stressed cultures as a percentage of the values of the control cultures (without metal addition), different strains and conditions could be compared. The graphs in Figure 2 depict these maximal OD values for *S. oneidensis* MR1 and isolates MB4, FB18 and FS8 against the logarithm of total added cobalt (A), zinc (B), cadmium (C) and copper (D). Total concentrations of heavy metals tested were 25, 75, 150 and 400μM. Growth of *S. oneidensis* MR1 in the presence of 150μM cobalt (and higher) is inhibited, similar to results for FB18, whereas strains MB4 and FS8 appeared to be more sensitive to cobalt (Fig. 2A). Growth curves with zinc showed that *S. oneidensis* and MB4 ceased to grow at 150μM, while FB18 and FS8 continued to grow to higher maximal cell densities (Fig. 2B). However, these latter two isolates were relatively sensitive for cadmium (Fig. 2C). Toxicity of cadmium was also evident from the delayed growth by S. oneidensis at concentrations of 25μM and higher. In contrast, isolate MB4 was able to cope with concentrations up to 75μM Cd. Growth curves with varying copper concentrations (Fig 2D) showed that FB18 and FS8 were relatively sensitive to copper and that MB4 and MR1 were also affected by copper, but to a lesser extent.

The above described tests were conducted in an automated set-up using 100μl cultures growing on 10-fold diluted LB broth. Changing experimental conditions had considerable effects on metal-toxicity, as summarized in Figure 3 for different media (A), culture volumes (B) and pre-treatment of inoculum (C). Substantial growth was observed in presence of 750μM of copper, when cultivating *S. oneidensis* or strain MB4 on a medium rich in organic matter (0.6xfold diluted Luria Burtani broth (LB)) (Fig 3A). A minimal medium (MM), supplemented with 0.1% v/v yeast extract, similar to the medium used for anaerobic cultivation, only supported growth of strain MB4 at copper concentrations below 75μM. The ratio between culture volume and surface area was also important since maximal cell densities in 100ml were typically higher than measurements made in 25 or 100ml cultures (3B). Cultures of MB4 were exposed to 50μM copper (designated MB4*), and subsequently incubated in toxicity trials with 0, 5, 10, 15 and 50μM of copper (Fig. 3C). Somewhat lower growth yields were observed with copper concentrations below 15μM. However, copper-exposed cultures did show higher cell density and shorter lag-phase when challenged with concentrations above 15μM Cu, compared to the controls (14 and 19 hrs, respectively for control and 50μM Cu).



^ Figure 2: Maximal optical density obtained of aerobic cultures of *Shewanella oneidensis* MR1, isolate MB4, FB18 and FS8 in presence of varying concentrations of cobalt (A), zinc (B), cadmium (C) and copper (D) measured after 48 hrs and expressed as percentage of OD of control culture without metal addition. Experiments were carried out at least in triplicate in 100µl culture volumes with 10-fold diluted LB medium (standard deviation below 1%, error bars not visible).



> Figure 3: Maximal optical density obtained of aerobic *Shewanella* cultures grown in presence of copper and in medium with different nutrient load (A), in different culture volumes (B), and with and without pre-incubation with copper (C) measured after 48 hrs and expressed as percentage of OD of control culture without metal addition. A: growth of strains MR1 and MB4 in different dilutions (0.6, 0.4 and 0.1) of Luria Burtani (LB) broth (e.g. MR1_0.6xLB indicates strain MR1 grown on 0.6-fold diluted LB broth), and growth of MB4 on minimal medium (MM), as a function of the copper concentration (25-750µM). B: growth of strain FB18 in 100ml, 25ml and 100µl culture volumes and 25-400µM Cu. Error bars are standard deviation between duplicates for 25 and 100ml. C: growth of strain MB4 with copper (5-50µM) compared to growth of culture MB4* (inocula from pre-incubated culture grown in presence of 50µM Cu), conducted in minimal medium supplemented with 0,1% yeast extract and peptone, Except otherwise noted measurements were made in 100µl, further details as for figure 2

Copper toxicity tests with fumarate, iron- or manganese-oxides as electron acceptor

Shewanella oneidensis MR1 and the three isolates were all able to grow anaerobically with lactate as carbon source and fumarate as electron acceptor. As depicted in Figure 4, final biomass yield of *S. oneidensis* decreased at 75μM and higher concentrations of copper. Isolate MB4 appeared less sensitive to copper; low copper (25-75μM) resulted in higher maximal cell densities than obtained under control conditions, and at 150μM final growth yield was still considerable. Strains FS8 and FB18 were very sensitive to copper under these conditions as little growth was detected at 75μM.

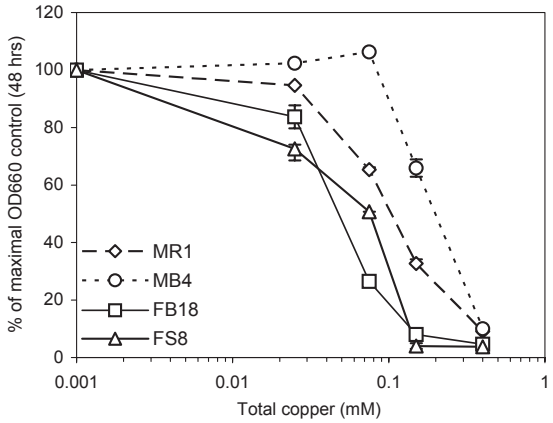


Figure 4: Maximal optical density obtained of anaerobic cultures of *Shewanella oneidensis* MR1, isolate MB4, FB18 and FS8 in presence of varying concentrations of copper and under anaerobic growth conditions with fumarate as terminal electron acceptor, measured after 48 hrs and expressed as percentage of OD of control culture without metal addition. Experiments were carried out in duplicate with 20ml of minimal medium, 0,1% yeast extract, 10mM fumarate and 20mM lactate.

All tested strains are able to reduce iron-hydroxides, but in contrast to MR1 and MB4, strains FB18 and FS8, isolated with HFO as electron acceptor, are unable to reduce Mn(IV). These latter two isolates showed very limited or no growth when offered iron-hydroxides (30mg dry wt) supplemented with 25μM of copper (1.6mg/l), compared to control cultures. Strains MR1 and MB4 vigorously reduced Fe(III) at this copper concentration, but did not show growth at higher concentrations. When strains MB4 and MR1 were grown under Mn(IV)-reducing conditions with excess of lactate, Mn(IV) oxide was reduced completely, although a very small amount of whitish brown precipitate remained. Copper toxicity during Mn(IV)-reduction was dependent on concentration of manganese oxides; final concentrations of 1mM Cu (63mg/l) did not limit growth of isolates MB4 and MR1 in the presence of high amounts of δ–MnO_2 (1.6g dry wt). Experiments involving lower amounts of manganese oxides (16mg dry wt) are described in a later section.

Figure 4 shows a compilation of microscopic images of strain MB4 under Mn(IV)-reducing conditions in presence of copper (5A-B) and higher resolution pictures made with a scanning electron microscope depicting δ–MnO_2 (5C) and the precipitate remaining after full microbial reduction (5D). Attached to large lumps of dark manganese oxides (contours depicted by dashed white line), several small, DAPI-stained, rod-shaped cells (white arrows) were visible in the initial growth phase in the presence of 500μM Cu (Fig. 5A). Phase-contrast images of later stages of growth (Fig. 5B) showed large flocks of microbial biomass (contours depicted by dashed white line), surrounding small, optically refractive, lemon-shaped particles (average diameter 6mm). The scanning electron microscope images largely confirmed the changes in size and morphology of δ–MnO_2 (Fig. 5C-D). With energy-dispersive x-ray spectrometry the Mn:O element ratio was determined at 0.44 in the original δ–MnO_2 and 2% atomic ratio copper. Similar analyses of the lemon-shaped particles that remained after microbial reduction indicated an enrichment in manganese (Mn:O ratio 0.60) and revealed that these particles no longer contained copper. All these observations were similar in Mn(IV)-reducing cultures without Cu (except, in addition to Mn and O, potassium, instead of copper was detected).

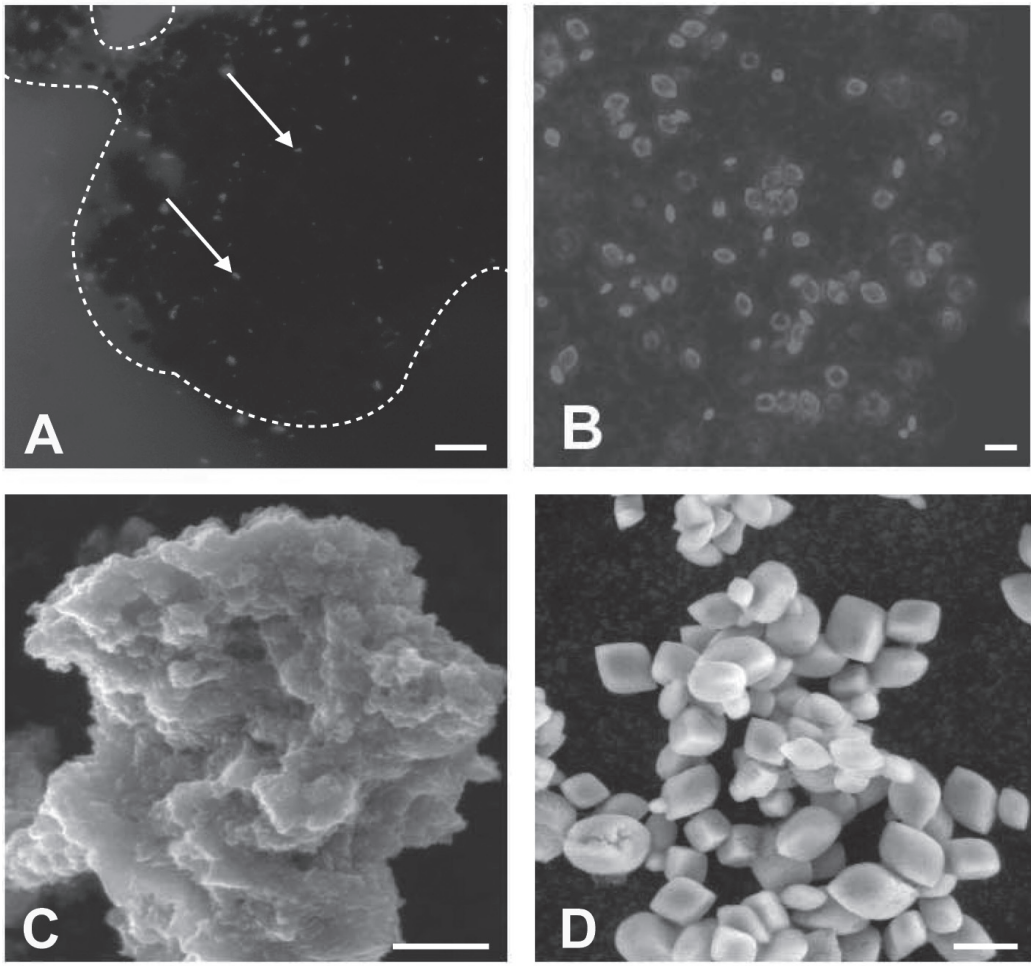


Figure 5: Compilation of microscopic images of isolate MB4 under Mn(IV)-reducing conditions in presence of 500µM copper (A, B), and more detailed scanning electron microscope images of δMnO_2 (C) and of the precipitate remaining after microbial reduction (D). All scale bars indicate 10 µm. A: against the dark shape of manganese oxides (white dashed line), DAPI-stained, rod shaped cells of MB4 are visible (white arrows) during initial growth , B: phase-contrast image of a cluster of MB4 cells, surrounding refractive, lemon-shaped particles, at end of exponential growth, C: SEM image of amorphous δMnO_2 before addition of cells and D: SEM image of product remaining after Mn(IV)-reduction by strain MB4.

Manganese oxides determine copper-availability and -toxicity to isolate MB4

The effect of copper adsorbed to manganese oxides during anaerobic growth was studied with isolate MB4 in a number of two-phased toxicity tests. Prior to these experiments, the maximal adsorption capacity (MAC) of copper to manganese oxides under the conditions applied in this study, was determined (0.14mg Cu/g oxide or 4.5mg Cu/g dry wt). Correspondingly, δMnO_2 was contaminated with copper at 0.5x, 1x and 25xMAC value, and offered to strain MB4 under Mn(IV)-reducing conditions. After all δMnO_2 had been reduced, the second phase was initiated by addition of fumarate to the same anaerobic batch-systems. Results of these copper toxicity experiments are summarised in Figures 6A-C.

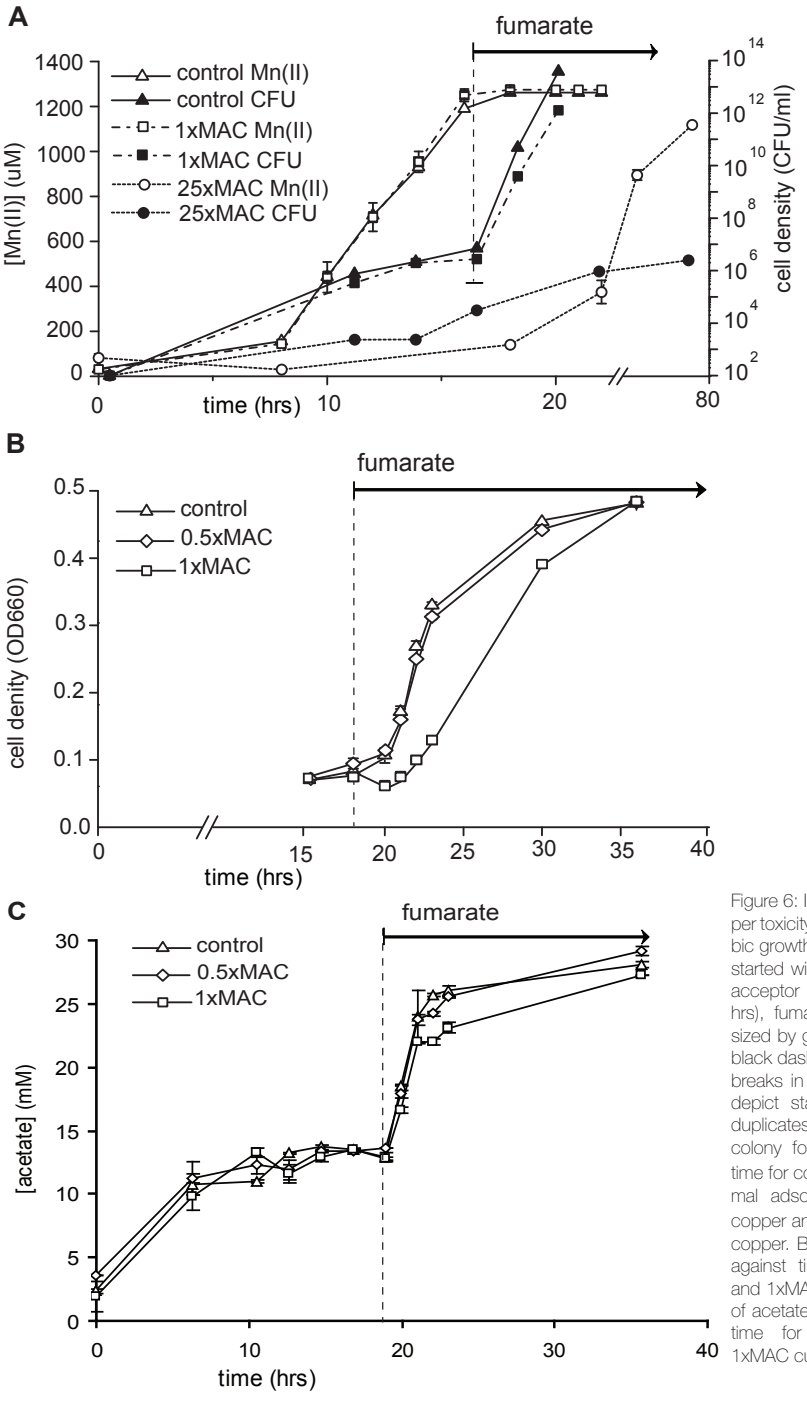


Figure 6: Influence of δMnO_2 on copper toxicity during two-phasic anaerobic growth of strain MB4. Experiment started with Mn(IV)-oxide as electron acceptor and after full reduction (17 hrs), fumarate was added (emphasized by grey triangle (A) and vertical black dashed line (B, C). Please note breaks in horizontal axes; error bars depict standard deviation between duplicates. A: Mn(II)-formation and colony forming units (CFU) against time for control, δMnO_2 with 1x maximal adsorption capacity (MAC) of copper and δMnO_2 with 25xMAC of copper. B: optical density at 660nm against time for control, 0.5xMAC and 1xMAC. C: concentrations (mM) of acetate in culture medium against time for control, 0.5xMAC and 1xMAC cultures.

Figure 6A shows Mn(II)-formation and colony forming units (CFU) plotted against time. Strain MB4 growing in the presence of δMnO_2 with 1xMAC copper, produced Mn(II) at the same rate as the control, whereas manganese reduction of 25xMAC is severely limited. In fact, only one of the duplicates reduced Mn(IV) completely and only after 72 hrs. The other replicate ceased growing after 40 hrs. This limited growth is confirmed by low CFU numbers relative to the control and 1xMAC. After fumarate addition (72 hrs) no further growth occurred in this batch. The control and 1xMAC cultures had completely reduced all manganese oxide in 17 hrs, based on the disappearance of the dark coloured oxide and also indicated by the plateau reached by the Mn(II) concentration. After the control and 1xMAC cultures had received fumarate (17hrs), CFU further increased. Measuring optical density after fumarate amendment, for control conditions, 0.5x and 1xMAC resulted in the graph shown in Fig 6B. The growth curve of 0.5xMAC is very similar to that for the control culture. Contrastingly, the 1xMAC culture showed an elongated lag-phase of ~3 hours, confirming the lower cell numbers suggested by CFU counts (Fig. 6A). Finally figure 6C shows measured concentration of acetate against time suggesting little difference between rates of acetate production between the control conditions, 0.5X and 1xMAC. Acetate increased from 3 to 13mM during Mn(IV) reduction and further growth with fumarate as electron acceptor raised concentrations to approximately 28mM

Discussion

Three metal-reducing strains were isolated from two marine sediments: FB18 and MB4 from a heavy metal-polluted harbour basin sediment and FS8 from a relatively undisturbed intertidal flat.16S rRNA sequences similar to isolates MB4 and FS8 were detected previously in DGGE profiles of the original sediment samples (accession numbers EU140511 and EU140512) and as minor constituents of the bacterial population in sediment HB according to clone library data (DQ334623, DQ334632 and DQ334633, chapter 3) (Fig. 1A). These results suggest that isolates MB4 and FS8 represented numerically relevant populations of Bacteria, confirming the wide-spread occurrence of members of the genus *Shewanella* in aquatic habitats (Ziemke et al., 1998; Ivanova et al., 2003), as well as the fact that they belong to the readily culturable bacteria. Interesting is the finding that the two strains obtained under iron-reducing conditions, FB18 and FS8, isolated from different sedimentary environments (regarding organic matter content, grain size, salinity, light etc), are similar in 16S rRNA (98% gene similarity) and *gyrase B* genes (99% gene similarity), and approximately equally sensitive (or tolerant) to the various heavy metals tested under different conditions.

Of the four tested strains, isolate MB4 was most tolerant to cadmium and copper under aerobic conditions. On the other hand, isolates FB18 and FS8 were more resistant to cobalt and zinc, when compared to *S. oneidensis* and MB4. Toxicity effects were evident from increased lag-phases, reduced growth-rates or a combination of both. Under anaerobic conditions with fumarate as electron acceptor, tolerance for copper was lower in isolates FB18 and FS8, but remained unchanged in strain MR1 and even increased slightly in strain MB4. This was unexpected, as decreased copper tolerance in cultures of other facultative anaerobes as *E. coli* has been demonstrated (Outten et al, 2001). Perhaps this is caused by the hypothetized, oxygen-dependent role that the *cueO* locus plays in copper resistance in *E. coli*, and the fact that no homologue was detected in the *S. oneidensis* genome. As differences existed between the applied culture media, volume and containment-material (e.g. glass or plastic) existed, reliable comparison of metal toxicity under different conditions remained difficult.

Maximal metal concentrations (Co, Zn, Cd and Cu) at which growth was observed with isolates MB4, FB18, FS8 and type-strain *S. oneidensis* MR1 presented in this paper were in the micromolar range under all tested conditions, in contrast to values published for *E. coli* and *C. metallidurans*, which are in the millimolar range (Nies, 1999). Although nutrient composition and organic matter content of the media used in the latter studies may have had some compensatory effects, in comparison the tested *Shewanella* strains were metal-sensitive. The range of tolerated metal concentrations values determined here for MB4 and FB18 did, however, correspond to metal concentrations measured in the harbour basin sediment, where they were isolated from (Tankere-Muller et al, 2007). The most abundant heavy metal detected in pore-water was copper with concentrations up to 400nM; it should be noted, that the highest labile copper and cadmium concentrations were detected at the sediment surface, suggesting metal release through aerobic organic

matter oxidation rather than metal-oxide reduction. However, maximal cobalt concentrations did coincide with the location of manganese solubilization, presumably by microbial reduction. It is conceivable that in this harbour sediment strain MB4 is partially responsible for the observed mobilization of cobalt by reducing the Mn(IV) oxides, in a process similar to the one demonstrated here for copper.

Our results furthermore indicate that the presence of manganese oxides decreases the bio-availability of copper through sorption process, thereby alleviating the toxicity of copper to Bacteria. Although HFO is assumed to play a similar role, cultures grown under iron-reducing conditions were more susceptible to copper. The protective effect of manganese oxides was dependent on the ratio copper: δ-MnO_2. Both strains MB4 and MR1 showed unaffected Mn(IV)-reduction rates in presence of 1mM copper and excess Mn-oxides (decreasing bio-available copper to a minimum). By preparing δ-MnO_2 with copper at concentrations above, around and below threshold values of maximal adsorption capacity, we showed that the onset of copper toxicity to isolate MB4 shifted from the first phase (Mn(IV)-reducing) to the second phase (fumarate). At the highest copper concentration tested, toxicity started immediately, as evidenced by slow Mn(II) formation rates and low cell numbers. When copper was added at values approximating maximal adsorption capacity, no changes were observed in the first phase but an increased lag-phase was evident after addition of fumarate as electron acceptor, suggesting that copper was released of the metal-oxides after full microbial reduction. Applying copper concentrations well below maximal adsorption capacity indicated no significant toxicity effect in either phase. Scanning electron microscopic images showed the initial amorphous Mn(IV)-oxides and concomitant electron dispersive x-ray spectrometry confirmed presence of attached copper. The gradual release of Mn(II) during bacterial reduction by MB4 or MR1 and subsequent partial re-precipitation, possibly as $Mn(II)CO_3$, may be responsible for formation of the highly crystalline, lemon-shaped, particles. These particles no longer contained detectable copper. As no significant toxicity effects were observed before depletion of δ-MnO_2 in 0.5x and 1xMAC cultures, release of sorbed copper seemed to occur in an instant rather than gradually. This may indicate that mobilized copper re-precipitates to the solid phase as long as δ-MnO_2 is still available. The fact that acetate production rates were similar between control, 0.5x and 1xMAC cultures, and that the latter showed significantly lower cell numbers during the second phase (in OD and CFU), indicated an additional energy expenditure or uncoupling of growth and energy metabolism. This energy requirement can be explained by the need to actively extrude copper from the cell, which was released from δ-MnO_2 during the first phase of the experiment.

Analyses of the annotated genome of *S. oneidensis* revealed the presence of several genes encoding proteins known to be involved in copper transport; e.g. ATPases involved in active uptake or extrusion (SO_1689, SO_2359; COG2217, www.cnr.tigr.org) as well as heavy metal efflux pumps driven by proton motive force (SO_0520, SO_4598 and SO_A0153; COG3696). As noted by Heidelberg and co-authors, the genome of *S. oneidensis* contains fewer transition metal transporters in comparison with related bacteria with equally sized genomes as *E. coli, V. cholerae* and *P. aeruginosa* (2002). A finding that is particularly remarkable as metals play such an important role in the anaerobic lifestyle of *Shewanella*. Future work will focus on the expression of above mentioned genes involved in copper homeostasis in *S. oneidensis* and isolate MB4 in aerobic and anaerobic conditions.

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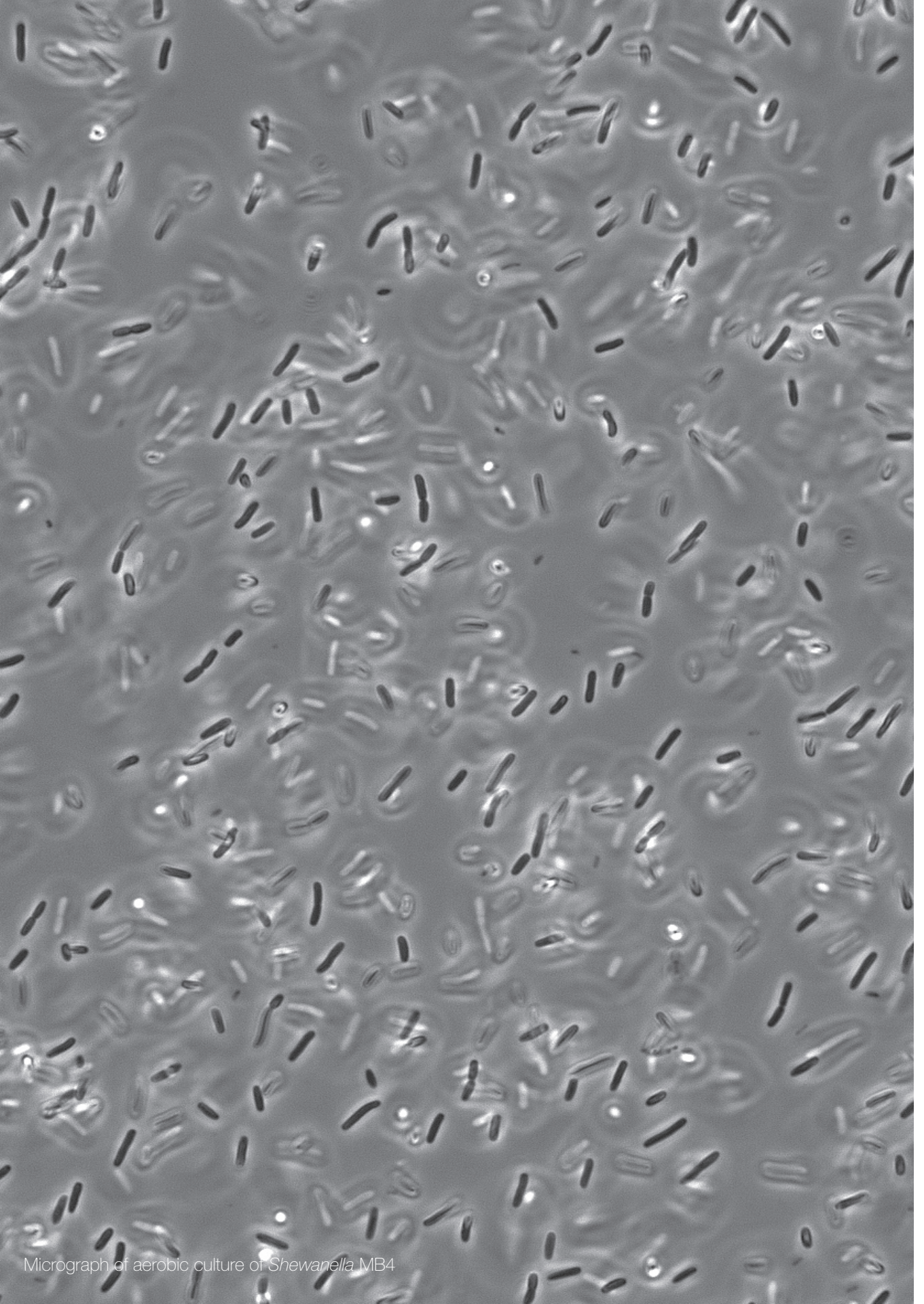

Micrograph of aerobic culture of *Shewanella* MB4

5 Expression of *copA* and *cusA* in *Shewanella* during copper stress

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Abstract

Copper homeostasis is tightly regulated in all living cells as a result of its necessity and toxicity in free cationic form. In Gram-negative Bacteria CPx-type ATPases (e.g. copA in *E. coli*) and heavy metal efflux RND proteins (e.g. cusA in *E. coli*) play an important role in transport of copper across the cytoplasmic and outer membrane. In the present study we investigated the expression of *cusA*- and *copA*-like genes in *Shewanella oneidensis* MR1 and *Shewanella* strain MB4, a Mn(IV)-reducing isolate from a metal-polluted harbour sediment. Q-PCR analysis of total mRNA extracted from cultures grown under aerobic conditions with 25μM of copper showed significantly increased expression of *cusA* (student's t-test, MR1: $p<0.0001$ and MB4: $p=0.0006$). This gene was also induced in presence of 100μM of copper and 10 or 25μM of cadmium in both tested strains. In absence of oxygen, with fumarate as final electron acceptor and 100μM copper, a prolonged lag-phase (5hrs) was observed. and general fitness decreased as evidenced by 2-fold lower copy-numbers of 16S rRNA compared to aerobic conditions. *CusA* expression in cells grown under these conditions remained at comparable levels (MR1) or slightly decreased (MB4), compared to aerobic copper challenges. A gene homologous to the *copA* gene of *S. oneidensis* was not detected in strain MB4. Although low levels of *copA* copy-numbers were observed in strain MR1 under conditions with 25 and 100μM of copper, *copA* was not detected in mRNA from cultures grown in presence of 10μM of cadmium, or in absence of metals. However, *copA* was highly induced under anaerobic conditions with 100μM of copper ($p=0.0011$). This data suggests essentially different roles for the two proteins copA and cusA in the copper response in *S. oneidensis* MR1, similar to findings in more metal-resistant bacteria as *E. coli* and *C. metallidurans*.

Introduction

Copper is required for growth in all living organisms, yet becomes toxic at higher concentrations. Therefore, transport of copper atoms across the cell membrane and subsequent incorporation into metalloproteins is tightly controlled (Rae et al., 1999; Nies, 1999). Two types of proteins involved in bacterial heavy metal efflux (HME) are CPx-type ATPases and HME-RND proteins (from the Resistance, Nodulation and cell Division protein family) (Paulsen et al., 1996; Coombs & Barkay, 2004, 2005; Saier, 2003). CopA in *E. coli* was among the first heavy metal-transporting ATPases to be fully characterized and showed transport of Cu(I) out of the cytoplasm at the costs of ATP hydrolysis (Rensing et al., 2000). Other relevant examples include cadA and zntA, involved in export of Zn^{2+}, Cd^{2+} and Pb^{2+} (Tsai et al., 2002; Banci et al., 2003; Gaballa & Helmann, 2003). CopA-like transporters are quite common and were detected in numerous micro-organisms (or their genomes), e.g. *E. hirae, Synechocystis, B. subtilis, S. mutans, Archeoglobus fulgidus, Ferroplasma acidiarmus* and lower eukaryotes as *Cryptosporidium parvum* and *Candida albicans* as well as mammals (Baker-Ausitin et al., 2005; Coombs & Barkay, 2005; Ettema et al., 2006;).

Examples of HME RND proteins are found only in Gram-negative Bacteria and include the czcCBA-system of the extreme metal-resistant bacterium *Cupriavidus metallidurans* (formerly known as *Ralstonia, Alcaligenes and Wautersia*), exporting divalent cobalt, zinc and cadmium, and the cusCFBA-system in *E. coli*, responsible for transport of monovalent copper and silver (Goldberg et al., 1999; Franke et al., 2003; Nies, 2003). These proteins are distantly related to well-studied multidrug transporters as AcrB in *E. coli* and MexB in *P. aeruginosa*, conferring resistance to acriflavin and other antibiotics. In contrast to ATPases, metal transport by RND proteins occurs in an antiport fashion, i.e. at the cost of proton motive force. Additionally, ATPases only span the inner membrane and transport from cytoplasm to periplasm or vice versa. RND proteins however, take up substrates from the cytoplasm (possibly also from the periplasm (Outten et al., 2001)) and transport these across both membranes to the cell's exterior. This suggests essentially different, although perhaps complementary functions in metal extrusion.

A number of studies have pointed at this apparent overlap in functions between different proteins in heavy metal resistance. In *E. coli*, the interactions between proteins from the cus (RND) and the cop (ATPAse) operons were investigated under copper stress (Outten et al., 2001; Kershaw & Brown, 2005). Expression of *copA* was observed under most tested conditions, whereas *cusA* was only induced at higher concentrations of copper (experiments carried out under both aerobic and anaerobic conditions). An additional gene, *cueO,* was associated with the physiological response to copper and hypothesised to convert Cu+ to the less toxic Cu2+ in the periplasm (under aerobic conditions). On the other hand, Legatzki and co-authors investigated the interplay between the czc system (RND) and two P-type ATPases in cadmium and zinc resistance of *C. metallidurans* (2003). In this bacterium, it was shown that the czc system alone was sufficient for removal of both metal cations under moderate levels of stress, but one of the ATPases (cadA) proved essential for full cadmium tolerance. More recently, transcriptional profiling of copper stressed *P. aeruginosa* indicated up-regulation of 2 RND efflux systems and an ATPase, as well a general fine-tuning of iron-acquisition (Teitzel et al., 2006).

Although newly annotated genomes suggest a wealth of genes encoding putative heavy metal efflux pumps, physiological evidence is only provided for a select few representatives. Members of the genus *Shewanella* represent interesting study objects in this sense, as many representatives are known for their capacity to reduce and detoxify a number of (toxic) metals (Nealson et al., 1992; Guha et al., 2003; Saltikov et al., 2003), yet little is known about their resistance to copper. In addition, *Shewanella* sp. have been isolated from (and detected in) a wide range of aquatic habitats, including relatively pristine and polluted environments (Ziemke et al., 1998; Ivanova et al., 2003). Enrichments for Mn(IV)-reducing bacteria from a heavy metal-polluted harbor sediment led to isolation of a *Shewanella* strain, designated MB4 (98% similarity to *S. marisflavi* based on 16S rRNA and *gyrB* gene). Physiological responses of isolate MB4 and *S. oneidensis* to the heavy metals cobalt, cadmium, zinc and copper under varying growth conditions are described elsewhere (chapter 4). Copper tolerance of both strains in presence of oxygen, evidenced from lag-phase and growth-rate, did not change dramatically during anaerobic growth on lactate and fumarate, in contrast to results obtained for *E. coli* (Outten et al., 2001).

In this study we report the detection of copA- and cusA- in mRNA of S. oneidensis and strain MB4 in response to copper, monitored with Quantitative-Polymerase Chain Reaction (Q-PCR). Cultures of S. oneidensis and isolate MB4 were subjected to copper-challenges and cells harvested for mRNA extraction at different points during the growth cycle. Reproducibility between different cultures was tested and results used to design an optimal experimental set-up for comparison between different stress conditions. These included, in presence of oxygen: 25μM and 100μM of copper and 10-25μM of cadmium. In addition, the 100μM copper trial was conducted under anaerobic growth conditions.

Experimental procedures

Strains and cultivation conditions

Shewanella oneidensis MR1 was kindly provided by K. Nealson, from University of Southern California, Los Angeles, USA. Strain MB4 was isolated from metal-polluted, marine harbour sediment under Mn(IV)-reducing conditions (97% 16S rRNA gene similarity to *S. marisflavi*). During a practical course at the department of Biotechnology (Delft university of Technology), a photoluminscent, *Vibrio*-like bacterium was isolated from the skin of North Sea fish (identified as 97% similar to *V. alginolyticus*). From the Netherlands Culture Collection of Bacteria (Utrecht) we obtained the following cultures: *E. coli* K12 (MG 1655 NCCB 4007), *Cupriavidus necator* (formerly known as *Ralstonia eutropha* H16, NCCB 82042) and *Bacillus azotoformans* (NCCB 100003).

All cultures were kept as glycerol stocks at -80°C. Each test was carried out with cultures that had not been previously exposed to heavy metals. Each culture was transferred three times to new medium after approximately 8 hrs of growth, prior to the actual experiments. The growth medium used in the aerobic experiments consisted of 10-fold diluted LB broth (per liter: 1g tryptone, 0.5g yeast extract and 5g NaCl) with extra salt added for MB4 (27.5g total). The minimal marine medium used for anaerobic tests contained per liter (g): 1 NH_4Cl, 0.2 $MgSO_4.H_2O$, 0.1 $CaCl_2.2H_2O$, 0.05 $K_2HPO_4.3H_2O$, 27.5 NaCl (5 for strain MR1) 0.5 yeast extract and 2.38 HEPES (pH 7.5). The medium was divided in 60ml portions in glass flasks (100 ml), closed with butyl rubber stoppers and the gas-phase was flushed with argon gas. After sterilization lactate (20mM), fumarate (10mM) and iron (20μM) were aseptically added from anaerobic stock solutions. Flasks were incubated at room temperature in the dark. Growth was monitored by microscopy and measuring the optical density at 595 nm.

Primer design

Primers for amplification of *copA*-like genes from members of the genus *Shewanella* were designed using multiple protein sequence alignments. *S. oneidensis* MR-1 '*copA*'-like protein (NP_717300, http://www.ncbi.nlm.nih.gov) was queried against the KEGG GENES Database (Kyoto Encyclopedia of Genes and Genomes; http://www.genome.jp/kegg/genes). Top matching sequences from several other representatives of the γ-subclass of the Proteobacteria were retrieved and used to generate a multiple sequence alignment in ClustalW (Lassmann & Sonnhammer, 2006); http://www.ebi.ac.uk/clustalw/index). This alignment was imported into the primer design tool CODEHOP to suggest primers (http://www.blocks.fhcrc.org/blocks/codehop, Rose et al, 2003). Secondary structures were predicted using NetPrimer (Premier Biosoft International, Palo Alto, CA, USA; http://www.premierbiosoft.com/netprimer/netprlaunch/netprlaunch) with default settings. Potential primers were blasted against all bacterial genomes with the BLASTN search for short, nearly exact matches to avoid sequence similarities with other than those encoding the desired genes. Potential primers were tested in silico in closely and distantly related bacterial genomes (Bikandi et al, 2004; http://www.insilico.ehu.es/). Several primer sets were tested in vitro on target and non-target DNA (see next paragraph).

A similar strategy was followed to design primers for the genes encoding 'cusA'-like proteins of *S. oneidensis* (NP_720114 and NP_720469) and related sequences. However, the obtained primers resulted in formation of an unspecific, larger amplicon in PCR reactions with *E. coli* control DNA. The second attempt involved a more topological approach; the twelve transmembrane spanning helices

(TMH) and the two large periplasmic loops (LPL) were detected in the amino acid sequence with help of the predictive tool TMHMM (Sonnhammer et al, 1998; http://www.cbs.dtu.dk/services/TMHMM/). As the LPL regions are possibly involved in metal-binding and show relatively high sequence variability (Goldberg et al., 1999), new primers specifically targeted the second loop between helices VII and VIII. Consensus primers were designed from the nucleotide sequences for *cusA* in *S. oneidensis* and its two marine relatives *Shewanella baltica* OS155 and *Shewanella frigidimarina* NCIMB 400 with Primer3 (Rozen & Saletsky, 2000; http://frodo.wi.mit.edu./cgi-bin/primer3/primer3). Secondary structures and in silico specificity were analysed as described above.

Primer specificity

Specificity of primers was tested with polymerase chain reaction (PCR). All amplifications were carried out with the taq PCR Master Mix kit (QIAGEN, Germany) in a Thermocycler (BioMetra, Germany). Each reaction contained 1X taq PCR Master Mix, forward and reverse primers with final concentrations of 1 μM each, PCR grade water and approximately 50ng template DNA. Sequences of forward and reverse primers copA_F and copA_R are shown in Table 1. Positive controls were DNA from *S. oneidensis* and *V. alginolyticus*, and negative controls were DNA from *B. azotoformans, E. coli* and *R. eutropha*. PCR reaction included an initial denaturation step (95°C for 5 min), followed by amplification: 95°C for 1 min, 59°C for 1 min, 72°C for 30 sec (30 cycles), and a final extension at 72°C for 10 min. Sequences of forward and reverse primers cusA_F and cusA_R are shown in Table 1. Positive controls were genomic DNA from *S. oneidensis* and MB4, and negative controls were DNA from *R. eutropha* and *B. azotoformans*. PCR reaction for *cusA* was identical to program described above, except optimal annealing temperature was determined at 57°C.

Universal primers 1055F and 1392R were used for amplification of bacterial 16S rRNA genes (Ferris et al, 1996). All primers were purchased from Thermo Electron GmbH (Ulm, Germany). Visualisation of PCR products was carried out with gel electrophoresis on 2% (w/v) agarose gels (80 V for 30 minutes). Molecular marker GeneRuler 100bp DNA Ladder (Fermentas GmbH, Germany) was used for size confirmation. After electrophoresis gels were stained with ethidium bromide and visualization of bands was performed with the Bio-Rad Gel Doc 1000 system under UV illumination.

Table 1: Primers used for amplification and quantification of *cusA*, *copA* and bacterial 16S rRNA genes.

Target	Name[1]	Sequence (5' – 3')	Size (bp)	Reference
copA	copA_F	CGC CAC CAT GAA CAA CAT GAA RCA RAA YYT[2]	151	This study
	copA_R	GTA ATG GAA GAC AGT GCC ATI GCI GC[2]		
cusA	cusA_F	ATG ACG AAT GGC GTG AAG G	112	This study
	cusA_R	GAT ACG GGT TTT GAT GGG TTG		
16S	1055F	ATG GCT GTC GTC AGC T	352	Ferris *et al.*, 1996
	1392R	ACG GGC GGT GTG TAC		

[1] F = forward primer, R = Reverse primer

[2] R = A or G, Y = C or T, I = inosine

Cloning, sequencing and phylogenetic analysis

Specificity of the primers was confirmed by sequencing. PCR products of *cusA* amplified from *S. oneidensis* and MB4 genomic DNA, and of *copA* amplified from *S. oneidensis* and *V. alginolyticus*, respectively, were extracted from 2% agarose gels and purified with the QIAquick Gel Extraction kit (QIAGEN, Germany). DNA sequencing was performed with the appropriate forward primers by BaseClear (Leiden, the Netherlands).

In order to quantify *cusA* and *copA* during different experiments, the obtained PCR amplicons were cloned in *E. coli*, and resulting plasmids used as molecular standards in Q-PCR. PCR fragments amplified from *S. oneidensis* genomic DNA were purified as described above and individually cloned into pCR 2.1-TOPO TA cloning vectors and transformed into chemically competent TOP 10 *E. coli* cells according to manufacturer's manual (Invitrogen, the Netherlands). Plasmids with *cusA* and *copA* inserts were extracted using the QIAprep Spin Miniprep kit (QIAGEN). Quality check and concentration measurements were performed with NanoDrop, and the plasmids stored aliquots in -20°C until use.

Protein and nucleotide sequences were compared to sequences stored in different nucleotide and protein databases, as described under primer design. Selected sequences were used to generate alignments in clustalW (Lassmann & Sonnhammer, 2006), which were imported into Mega3 (Kumar et al., 2004). Final Neighbour-Joining trees were created with Poisson and Kimura correction, for proteins and nucleotides, respectively.

RNA extraction and quantification

Cell numbers were determined with optical density measurements at 590 nm prior to harvesting cells. 1-3ml of culture was taken aseptically, centrifuged at 1000rpm for 25 min, cell pellets resuspended in RNAlater (Ambion) and used directly or stored at -20°C for 2 days before further treatment. Care was taken to start RNA extraction with approximately equal amounts of bacterial cells, but (well) below maximal loading capacity of the used columns (7.5x108) of the RNeasy Mini Kit (Qiagen). RNAlater was removed by centrifugation and cells were lysed in TE buffer (10mM TrisCl, 1mM EDTA, pH8) containing 1mg/ml lysozyme for 5 minutes at room temperature. RNA was stabilized in RLT buffer containing β-mercapto ethanol (from the extraction kit) and subsequently purified on columns. DNAse treatment performed in waterbath (10 minutes, 37°C) with 1-2µg RNA in Dnase buffer (50mM Tris-Cl, pH8, 5mM $MgCl_2$) with Dnase I (0.5 Kunitz units). After this incubation, 2µl 140mM EDTA was added at 65°C for 5 minutes, and inactivated enzymes were removed. This protocol showed less RNA loss compared to on-column DNA digestion. With Nanodrop measurements before and after DNase treatment total extracted RNA was quantified, which enabled normalization of the amount of input RNA into the cDNA synthesis reaction. Only samples with good quality, i.e. 260/280 ratio ~2.0 (Fleige & Pfaffl, 2006), were considered acceptable for further processing, with the BioRad iScript cDNA synthesis kit. Control PCR reactions with 16S primers were carried out to check for DNA contamination, and cDNA dilutions of unknown samples were amplified to confirm equal target concentrations.

Q-PCR and statistical analyses

Amplification and detection of *czcA* and *copA* genes and bacterial 16S rRNA genes were performed with the iCycler detection system (Bio-Rad Laboratories, Inc.). Approximately 50ng cDNA was added to each reaction, based on amount of total extracted RNA and for 16SrRNA 5ng target. Data analysis was performed with the iCycler iQ Software. All reactions were performed in 25µl reactions and utilized the QuantiTect SYBR Green kit (QIAGEN GmbH). All PCR runs included triplicates of unknown samples, no template controls, and standard DNA in 10-fold dilution series. After amplification, melting curve analysis was performed to confirm amplification specificity. This was carried out in 80 cycles with 10 seconds per cycle, starting at 55°C and increasing 0.5°C per cycle. Q-PCR products were also analyzed with gel electrophoresis to check the amplicon size and detect primer-dimers (2% w/v agarose). Threshold cycle values and the log starting quantities for standards in real time PCR assays were used to obtain standard curves from which target concentrations in unknown samples could be calculated. Detection limits and valid template DNA input ranges were determined by performing real time PCR runs with dilution series of standards and samples prior to quantification runs. The unpaired student's t-test was used to determine whether two data sets were significantly different ($p<0.05$).

For *copA* amplification the master mix was diluted with PCR grade water and 0.6μM copA_F primer, 0.3μM copA_R primer, 0.08mg/ml BSA, 4.5mM MgCl and DNA template were added. The PCR program included a hot start activation step of 1 cycle at 95°C for 15min, followed by 30 cycles of 95°C for 1min and a combined annealing/extension step with fluorescence detection at 57°C for 30 seconds. For *cusA* amplification both primers were added to a final concentration of 0.3μM. Other conditions and reagents were similar to Q-PCR of copA, except that the annealing temperature was 59°C. For amplification of fragments of the16S rRNA genes an identical reaction mixture was used. The Q-PCR program started with 95°C for 15min, following 27 cycles of 95°C 20sec, 55°C 30sec and 72°C 1min. Fluorescence was measured in the extension step.

Results

Primer design for cusA and copA in *Shewanella*

Analyses of the annotated genome of *S. oneidensis* revealed several sequences with high similarity to genes encoding proteins involved in copper transport in other Bacteria; i.e. CPx-type ATPases involved in active uptake or extrusion (SO_1689 and SO_2359) as well as HME RND proteins driven by proton motive force (SO_0520, SO_4598 and SO_A0153). Comparative analysis of amino acids sequences with other *Shewanella* type strains and with bacterial reference sequences (from the α- and γ-subdivisions of Proteobacteria and from the *Firmicutes*) resulted in the phylogenetic trees shown in Figures 1 (ATPases) and 2 (RND proteins).

Two putative copper CPx-type ATPases were found, located in different regions of the chromosome. One of them, encoding protein NP_717949, was phylogenetically most closely related to known copper importers (Figure 1, designated cluster copA1). The other sequence (NP_717300) showed higher similarity to proteins in cluster copA2, involved in either copper export or import, as copA from *Enterococcus hirae* and *Staphylococcus aureus* (Solioz & Stoyanov, 2003; Coombs & Barkay, 2005). The box with the dashed line indicates the target sequences for primer design (Table 1). Regarding HME RND proteins (Figure 2), three potential sequences were detected, two of which are encoded on the chromosome (NP_720114 and NP_716156) and one on MR1's megaplasmid (NP_ 720469). Two sequences from *S. oneidensis* showed resemblance to cusA (*E. coli*) and silA (*Salmonella typhymurium*), transporting monovalent Cu and/or Ag; The third protein was related to sequences in one of the clusters without immedi-

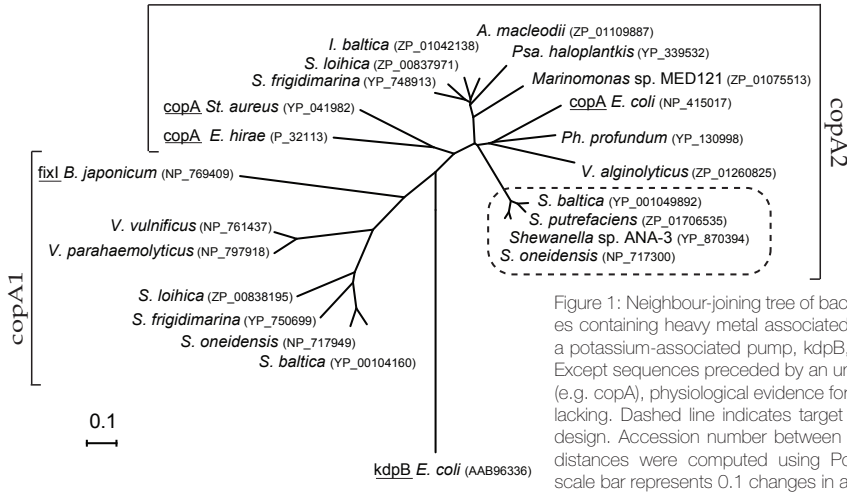
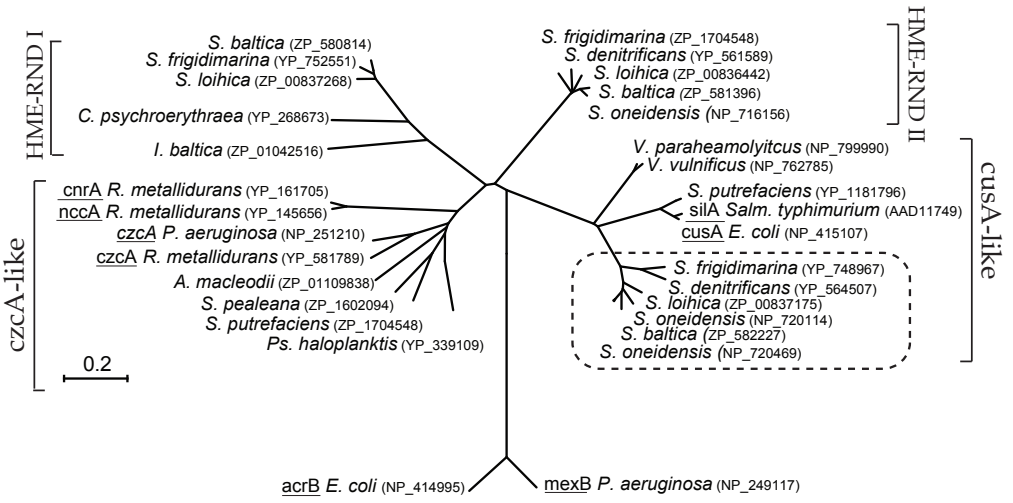
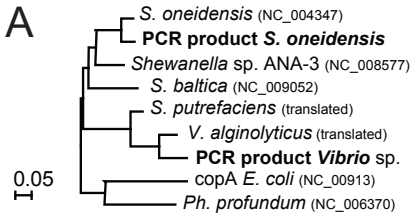
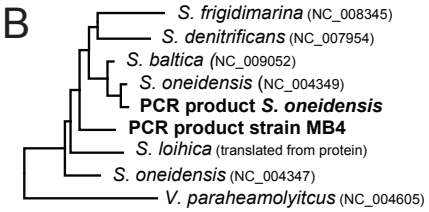


Figure 1: Neighbour-joining tree of bacterial CPx-type ATPases containing heavy metal associated domains, rooted with a potassium-associated pump, kdpB, from E. coli (750 aa). Except sequences preceded by an underlined protein name (e.g. copA), physiological evidence for transporter function is lacking. Dashed line indicates target sequences for primer design. Accession number between brackets. Evolutionary distances were computed using Poisson correction and scale bar represents 0.1 changes in aa.



^ Figure 2: Neighbour-joining tree of bacterial RND proteins involved in heavy metal efflux (HME), rooted with a multi-drug exporter, acrB, from *E. coli* (1050 aa). Except sequences preceded by an underlined protein name (e.g. cusA), physiological evidence for transporter function is lacking. Dashed line indicates target sequences for primer design. Legend as for Figure 1.





< Figure 3: Pruned neighbour-joining tree of nucleotide sequences obtained with novel primer sets copA (A, 130bp) and cusA (B, 110bp) on *S. oneidensis* and test strains. **Bold** type indicates sequences from this study. Scale bar represents 0.05 changes in both figures.

ate representatives, or knowledge about metal specificity (HME RND II). None of the sequences showed significant similarity with czcA-like proteins, although other *Shewanella* type strains were represented in this cluster. The designed primers targeted the copper determinants encircled by the dashed line.

The novel primer sets created for this study are listed in Table 1. Up to three degeneracies were incorporated into the oligo-nucleotides, in order to target possible similar genes in isolate MB4, besides detection in *S. oneidensis*. The primers were also tested on *C. eutropha, E. coli, Bacilus azotoformans* and a *Vibrio*-like isolate (of which only the last gave a positive signal and only for the copA primer set). Figure 3A shows a phylogenetic tree of the short nucleotide fragments generated with copA-primers; PCR amplification of *S. oneidensis* and the *Vibrio*-like isolate provided expected products, but for MB4 no PCR product was detected. Amplification with the cus-primers (Fig. 3B) did give a positive signal with both isolate MB4 and for *S. oneidensis*.

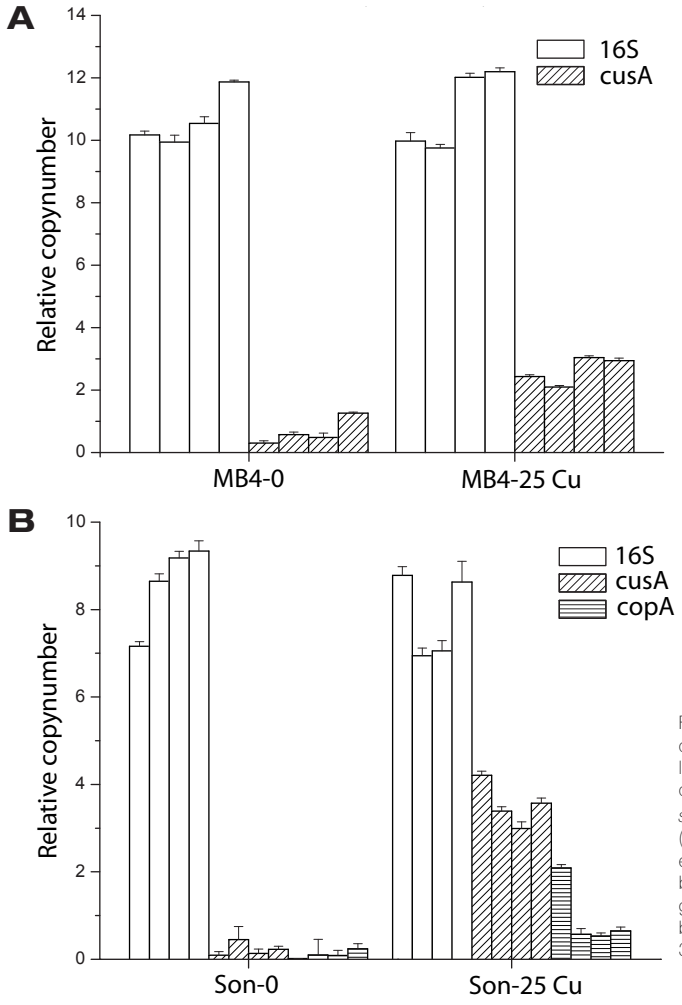


Figure 4: Comparison of Q-PCR (cusA, copA and 16S) results of 4 biological replicates from two separate experiments with cultures of isolate MB4 (A) and *S. oneidensis* (B) growing with or without 25μM copper (dosed at t=0 hrs and OD595<0.02, RNA extracted at OD 0.2). Relative copy-number is the natural logarithm of the calculated gene copy number per ng total RNA. Error bars represent standard deviation between 3 technical replicates.

Expression of *copA* and *cusA* during growth under aerobic and anaerobic conditions

In order to investigate whether these copper determinants showed increased expression under short-term copper stress, aerobic toxicity experiments were conducted. At different stages of growth, samples were taken for RNA extraction and subsequent quantification of *copA*, *cusA* and 16S fragments was conducted with Q-PCR (data not shown). Increased expression of the genes of interest in both *S. oneidensis* and isolate MB4 was detected in response to copper. Induction was dependent on copper dosage and time of sampling; *copA* and *cusA* rose above detection level within 2hrs after dosage, but the gradual decrease observed in the amount of 16S mRNA from 10^7 at OD0.05 to 10^3 at OD0.7 also indicated strong reduction of general fitness. In order to circumvent variability in growth phase and related mRNA production, further experiments were conducted with copper added directly at the time of inoculation (t=0hrs) and sampling at early log phase (OD 0.2). Additionally, these tests aimed to determine the reproducibility between measurements of 4 biological replicates, cultivated and analysed at two separate occasions.

Figure 4A shows the results of Q-PCR of *cusA* and 16S in mRNA of four biological replicates of strain MB4 growing in presence of copper (25μM) and control cultures. Similar experiments for *cusA, copA* and 16S rRNA gene quantification in *S. oneidensis* MR1 were conducted and results presented in Figure 4B. Relative copy-number is defined as the natural logarithm of the calculated gene copies, corrected for input mRNA (ng). Variation between biological replicates carried out at different occasions, was relatively

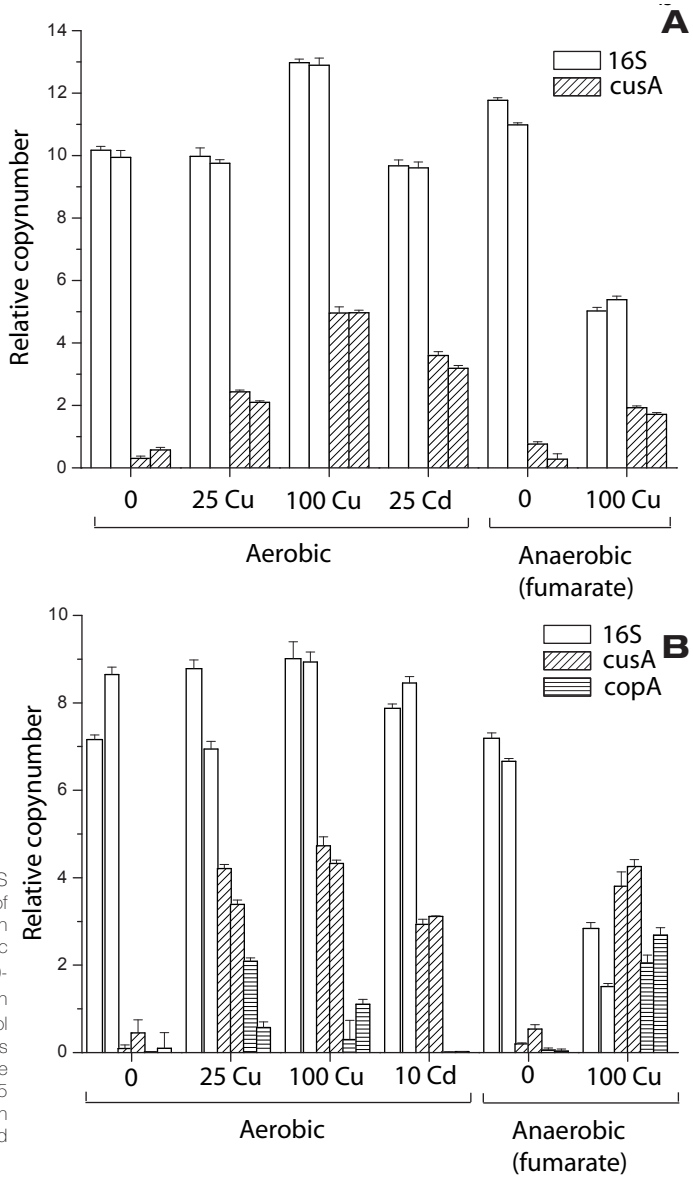


Figure 5: Quantification of cusA and 16S rRNA fragments in isolate MB4 (A) and of *cusA, copA* and 16S copy-numbers in RNA of *S. oneidensis* (B) during aerobic growth with 25μM Cu, 100μM Cu or 10-25μM Cd, during anaerobic growth with fumarate and 100μM Cu and control conditions (0). Copper or cadmium was present from OD<0.02 and cells were harvested at OD 0.2 (aerobic) and 0.15 (anaerobic). Experiment was carried out in duplicate and error bars indicate standard deviation between 3 technical replicates.

high for the genes investigated, 2-12%. For *S. oneidensis* the number of 16S rRNA copies per ng total mRNA ranged between $0.5*10^5$ to $2*10^5$. *CusA* quantification indicated 20-50 copies/ng mRNA during growth with 25μM copper, and even lower *copA* concentrations (0.5-5). The correlation between the presence of copper and expression of presumed copper determinant *cusA* was significant in MB4 and MR1 (unpaired student's t-test: $p<0.0006$ and $p=0.0001$). *CopA* expression in MR1 in presence of copper was not significantly different from control conditions under the tested conditions ($p=0.067$).

Final experiments were carried out, with the conditions described above, to determine differences in expression levels of *cusA* and *copA* under different degrees of metal stress during aerobic growth, i.e. with 25 and 100μM of copper and with 25μM of cadmium (10μM in case of *S. oneidensis*). Furthermore, growth with 100μM of copper was studied during anaerobic growth with fumarate as terminal electron acceptor. Figure 5 summarises Q-PCR results for cultures of isolate MB4 (A) and *S. oneidensis* (B) harvested at OD 0.2 (OD 0.15 for anaerobic cultures). The data presented in Figure 5A indicates that *cusA* expression increased approximately 2-fold between 25 and 100μM of copper, and was also sig-

nificantly induced under anaerobic conditions (p=0.038) and by cadmium (p=0.0013). From the data presented in Figure 5B, it is evident that *cusA* was detectable in MR1 under all conditions tested and that copy-numbers were significantly higher in cultures grown in presence of either copper or cadmium (aerobic, all p<0.0001, anaerobic 100μM: p=0.0059). Contrastingly, *copA* was only significantly induced under anaerobic conditions (p<0.001). Quantification of rRNA suggested that copy-numbers were similar throughout the experiment (in the range of 10^5 copies/ng), with the exception of samples from cultures grown under anaerobic conditions in presence of 100μM copper; both MB4 and MR1 produced significantly lower amounts of 16S rRNA, suggesting lower general fitness of the sampled cells.

Discussion

Copper efflux pumps encoded in the genome of *S. oneidensis* MR1

Although generally a high degree of variability is found among CopA sequences, there are a number of strongly conserved motifs. The two putative copper CPx-type ATPases encoded in the genome of *S. oneidensis* (Figure 1) contain the amino acid motifs characteristic for ATP binding (GDGIN), phosphorylation (DKTG), and phosphatase domains (TGE), common for all P-type ATPases (Rensing et al., 2000). In contrast to hard-metal ATPases which contain 10 transmembrane helices (TMHs), copper-transporting proteins consist of 8 helices. In addition, SxHP in the periplasmic loop and the metal-binding motif CPCAL in the 6th TMH indicate heavy metal-transporting ATPases (Coombs and Barkay, 2005). This cys-pro-cys sequence is essential for binding of Cu(I) as shown by site-directed mutagenesis of *copA* in *E. coli* (Fan et al., 2002). Despite an extensive genomic survey of copA-like sequences, Coombs and Barkay found no conserved motifs distinguishing proteins from the CopA1 and CopA2 clusters (2005). However, the vast majority of genes from the copA1 cluster was located adjacent to genes encoding the subunits of copper-heme oxidases, indicating a role in the supply of monovalent cations for metal-dependent enzymes. Likewise, in *S. oneidensis* the gene encoding NP_717949 (SO_2359) was located between a cytochrome C maturation protein and cytochrome C oxidase subunits (www.tigr.com). Primary sequence differences between the two ATPases were found in the N-terminal region. The targeted copA-like protein (NP_717300, SO_1689) contained one CxxC motif, whereas the ATPase in the copA2 cluster contained three repeats. According to Rensing and co-authors, these sequences encode cytosolic metal-binding motifs and may have regulatory functions (2000).

Less structural information is available for HME RND proteins (Paulsen et al., 1996). The roughly determined structure of CzcA in *C. metallidurans* as described by Goldberg and co-authors, is a protein with 12 TMHs and two large periplasmic loops and fits the model of a two-channel pump (1999). The highly conserved "DDE" motif in one of these channel (TMH IV), also found in multi-drug efflux pumps as ArcB, was essential for CzcA function in vivo and for proton/cation antiport in vitro. In RND systems involved in transport of monovalent copper and silver (cusA-like cluster in figure 2), a shorter DE motif is found. The DE motif was also present in all sequences from cluster HME-RND II. Three methionine residues at positions 573, 623 and 672 were shown essential for copper resistance by cusA in E. coli (Franke et al., 2003), and these were also detected in all sequences from the cusA-like cluster (not in HME RND II).

The *cus* determinant in *E. coli* encodes the cusCFBA complex, including a small open reading frame encoding cusF, a copper-binding periplasmic protein (Rensing & Grass, 2003; Franke et al., 2003). In *S. oneidensis,* the two putative *cusA* sequences were indeed found downstream of a membrane fusion protein ('cusB') but no '*cusF*'-like sequence could be detected. Instead small hypothetical proteins (86 aa), containing 2 putative copper-binding histidine repeats and a copper-transporting ATPase domain were found. It is likely that the plasmid encoded cusAB determinant (SO_A0153) is a recent duplication from the one in the genome (SO_4598), as the genes are organized in a similar way, share high sequence similarity and many transposons were identified in MR1's plasmid (Heidelberg et al., 2002; Kolker et al., 2005). The outer membrane protein ('cusC') may be encoded on a different location in the chromosome as was shown for acrAB and tolC. One putative outer membrane protein was detected upstream of the third HME RND sequence in cluster II (SO_0518) and may be recruited by the cusA-like protein systems.

Q-PCR of copA and cusA in *Shewanella* strains

A Q-PCR method was developed that allowed for detection of *copA*- and *cusA*-fragments in mRNA extracts of *S. oneidensis* and strain MB4. The 16S rRNA was also quantified as an indicator of physiological fitness. Although quantification of a reference gene or an internal standard may be one of the preferred methods for normalization of Q-PCR data (Sharkey et al., 2004), it is a laborious task to establish which reference gene itself is not regulated by the test condition. For instance, at least 200 protein-encoding genes of varying function are up- or down-regulated during the general stress response in *S. oneidensis* MR1 (Qiu et al., 2005), and similar effects may be expected in response to metal toxicity. Therefore, in this paper results were normalized by applying equal amounts of total RNA in cDNA synthesis and subsequent Q-PCR (Schmittgen & Zakrajsek, 20000; Hugget et al., 2005). Total RNA consists primarily of rRNA (80%), and only 2-5% is comprised of protein-encoding mRNA. Results from Liang and co-authors suggest that i) the number of available ribosomes is constant and independent of growth rate and ii) that efficiency of translation of mRNA is influenced by amount of bulk rRNA, due to competition for available ribosomes (2000). Therefore, care was taken to harvest cells at similar optical densities, ensuring comparable growth rates and rRNA production. Q-PCR results of four individually treated replicates of cultures harvested at OD600 0.2 (early exponential phase) indicated moderate reproducibility between biological replicates (2-12% deviation). This relatively high variation is not uncommon when compared to other studies using Q-PCR to assess in vivo gene expression (Vandecasteele et al., 2001; Nielsen & Boye, 2005). Reproducibility between technical replicates was high (0.1-0.8%).

Under aerobic growth conditions, the correlation between the presence of copper (25μM) and expression of presumed copper determinant *cusA* was significant in both MB4 and MR1 (unpaired student's t-test: $p<0.0006$ and $p=0.0001$). *CopA* expression in MR1 in presence of copper was not significantly different from controls under the tested conditions ($p=0.067$). Further aerobic tests pointed out that *cusA* expression in MB4 increased approximately 2-fold between 25 and 100μM of copper, and was also significantly induced by cadmium ($p=0.0013$). *CusA* was detectable in MR1 under all conditions tested and copy-numbers were significantly higher in cultures grown in presence of either copper or cadmium (all $p<0.0001$). When anaerobic growth conditions were applied and fumarate offered as terminal electron acceptor, *cusA* expression in presence of 100μM of copper was lower than under aerobic conditions, but still significantly higher than controls (MB4: $p=0.038$, MR1: $p=0.0059$). Interestingly, *copA* was only significantly induced under anaerobic conditions ($p<0.001$) in MR1.

A number of recent studies have specifically focused on *S. oneidensis* under different types of stress. Under UVA irradiation, multi drug transporting RND systems were strongly induced, in addition to both presumed *cusAB* operons in strain MR1 (7.8- to 14-fold) (Qiu et al., 2005). This may indicate that HME RND pumps function in detoxification of radiation products or are de-repressed as part of the general stress response. Transcriptome analysis of heat shock response genes, indicated no changes in the genes under study (Gao et al., 2004). Under Cr(VI)-reducing conditions, however, genes SO0518-SO0520 were up-regulated, suggesting that the sequences in cluster HME RND II may have a function in Cr(III) detoxification (Bencheikh-Latmani et al., 2005). A study of Groh and co-authors, showed that genes encoding RND proteins involved in antibiotic resistance are important determinants in the ecological fitness of *S. oneidensis* MR1 (2007). To add to this emerging picture, our results suggest that expression of *copA* and *cusA* in *S. oneidensis* mRNA extracts significantly increased during growth with copper. Furthermore, expression of *cusA* was observed under cadmium stress. *CopA* in particular appeared to play an important role in copper detoxification during anaerobic growth with copper. These findings are in contrast with results obtained for *E. coli*, which indicated that the copA protein was primarily expressed under aerobic conditions, whereas cusA was essential for full anaerobic copper tolerance (Outten et al., 2000; Kershaw & Brown; 2005). However, the operon encoding *copA* in *E. coli* also encodes *cueO*, a periplasmic oxygen-dependent oxidase. Perhaps, the absence of an oxygen-dependent multicopperoxidase such as *cueO* in *S. oneidensis* MR1 may help explain why copper resistance does not markedly change when comparing aerobic and anaerobic conditions (chapter 4). Increased *copA* expression in MR1 under anaerobic conditions results in active transport of Cu(I) from the cytoplasm to the periplasm, implying that an additional copper transporter may be involved to remove excess copper from the periplasm. As substantial expression levels of *cusA* were observed under these conditions, it is not unlikely that these HME RND protein systems are also capable of transporting metal cations from the periplasm, as suggested previously for CzcCB2A in *C. metallidurans* (Goldberg et al., 1999) and for cusCFBA in *E. coli* (Outten et al., 2000; Kershaw & Brown, 2005).

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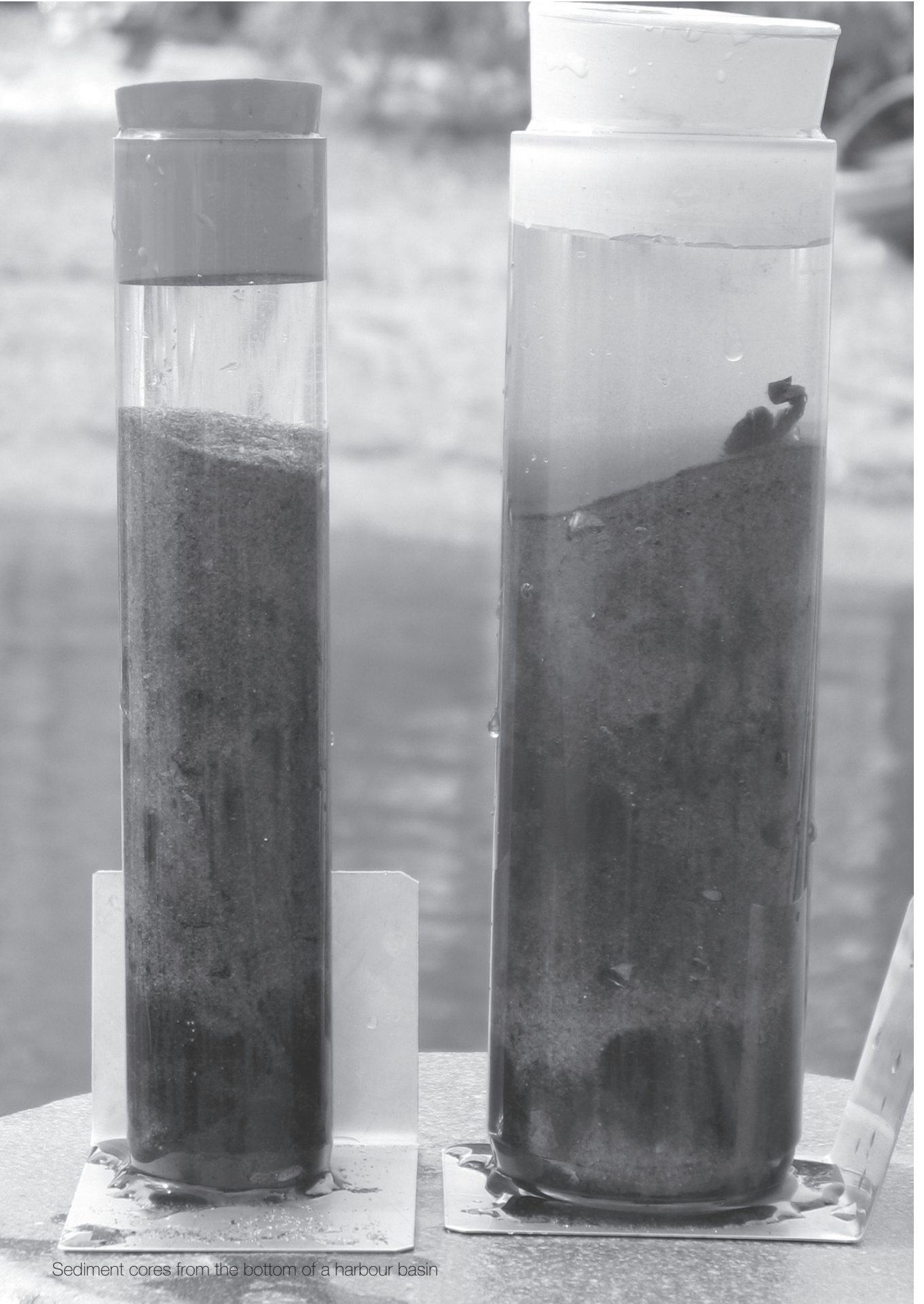

Sediment cores from the bottom of a harbour basin

6 *Shewanella copA* and *cusA* genes in marine sediments as potential indicators for bio-available copper

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Abstract

The goal of the present study was to investigate whether molecular quantification of microbial copper transporters could form a suitable strategy to assess bio-available copper in marine sediments. Primers designed to amplify fragments of copper transporters copA and *cusA* in *Shewanella oneidensis* MR1 and *Shewanella* MB4 (chapter 5), were applied in Quantitative-Polymerase Chain Reaction (Q-PCR). Microbial community DNA extracted from 11 different North Sea sediments was used as target in the Q-PCR assay; the strongest correlation was found between copynumber *cusA* and total extracted copper ($R^2 = 0,78$), but only when the 4 sandy sediments were excluded. Higher resolution analysis of microbial DNA extracted along a depth gradient (0,5cm interval) in one particular harbour sediment, revealed that the number of 16S rDNA genes decreased with depth from $1*10^{10}$ to $6*10^{9}$ per g of wet sediment. Recovery during DNA extractions was assessed by internal spiking of sediment samples prior to extraction, and yielded efficiencies of approximately 2%. *Shewanella copA* fragments ranged from 10-4400/g of wet sediment, and the *cusA* genes from 60-660/g of sediment. Statisical analysis of the Q-PCR results indicated that *cusA* and *copA* copy numbers were not uniformly distributed with depth, nor correlated with pore water copper concentrations or total extracted copper. Although the developed Q-PCR assay has extremely high specificity and initial results with the sediment collection looked promising, more reliable data on metal resistance genes, e.g. RNA-based measurements, are essential for proper estimation of bio-available copper.

Introduction

Microbial organisms have been exposed to heavy metals since the beginning of life and heavy metal determinants have evolved as a result of this exposure. However, industrial input of heavy metals into the environment has resulted in extensive pollution of many aquatic environments. Considerable metal pollution of sediments from harbours and marinas has been attributed to the application of antifouling paints on ship hulls of sea vessels (Schiff et al., 2004; Warnken et al., 2004). Heavy metals tend to accumulate in sediments, by precipitation as (hydr)oxides or metal-sulfides and/or by adsorbance to clay particles, humic acids, iron or manganese oxides organic matter etc. (Glasby & Schulz, 1999; Leipe et al, 2005). An important task in ecological risk assessments is to gain knowledge on the the bio-available fraction of heavy metals, since only dissolved metals in the form of "free" cations (e.g. Cu^{2+}, Zn^{2+}) are bio-available and thus toxic, whereas (in)organic metal complexes are not directly available for biological uptake (Worms et al., 2006).

As extrapolated from principles in macro-ecology, it is generally assumed that heavy metal pollution affects size and diversity of a microbial community (Sandaa et al., 1999ab; Worm et al., 2002; Li et al., 2006). However, some studies also demonstrated that environmental stress did not decrease microbial diversity, as judged from 16S rDNA analysis, but rather induced a shift in dominant species, while the total number of species remained approximately constant (Powell et al., 2003; Gillan et al., 2005, Yoshida et al., 2005). Instead of using the 16S rDNA genes for taxonomic purposes and diversity studies, other approaches directly targeted functional genes involved in the metabolic degradation of a pollutant, e.g. naphtalene (Dionisi et al., 2004; Nyyssonen et al., 2006), or targeted resistance genes as *cadA* for cadmium transport (Oger et al., 2003) or *tetG* for tetracycline (Yu et al., 2005). These types of studies give information about the capacity of a certain microbial population to cope with environmental pollutants. Additionally, some insight may be provided into the role of horizontal gene transfer in the acquisition of resistance genes within a microbial population. Our research aimed to investigate if a connection exists between the degree of heavy metal pollution, in particular copper, and the total number of heavy metal resistance genes in a microbial population.

Studies of plasmid ecology suggest that metal resistance genes are frequently transferred horizontally (Sobecky, 2002). Additionally, comparative genomics indicated that certain heavy metal transporting protein encoding-genes are abundant in genomes of Bacteria and Archaea (Coombs and Barkay, 2005). Two copper transporting proteins, copA and cusA were selected for this study based on previous research with *Shewanella oneidensis* MR1 and its available genomic sequence and a number of marine Shewanella isolates (chapters 4 and 5). CopA, a copper-transporting ATPase, was first characterized in *E. coli*, transporting Cu(I) out of the cytoplasm at the costs of ATP hydrolysis (Rensing et al., 2000). Whilst *copA* genes are quite abundant among both prokaryotes and eukaryotes, *cusA* genes are found only in Gram-negative Bacteria. These multi-subunit protein complexes (e.g. cusCFBA-system in *E. coli*) belong to the Resistance, Nodulation and cell Division (RND) family of proteins and transport monovalent copper and silver in an antiport fashion (Goldberg et al., 1999).

A Quantitative-Polymerase Chain Reaction (Q-PCR) based approach was developed to detect and calculate *copA* and *cusA* copy numbers, in *Shewanella oneidensis* MR1 and a closely related marine isolate, strain MB4. Initial tests aimed to investigate the effects of DNA target concentrations and co-extracted PCR inhibitors on amplification efficiency. In order to determine the target specificity of the primers, DNA extracted from an industrial harbour sediment was used as template in PCR. The amplification products were cloned in *E. coli* and inserts of 10 randomly selected clones sequenced. Subsequently, DNA was extracted from 11 different North Sea sediment samples with known metal-concentrations, and copy numbers of 16S, *cusA* and *copA* genes were determined with the Q-PCR method. In addition, one harbour sediment was studied in more detail, by sampling along a 5mm-scale depth profile. A possible correlation between detected copper concentrations and *cusA* and/or *copA* copy numbers would be a first step in the development of a new type of strategy for estimating bio-available copper in marine sediments.

Experimental procedures

Site descriptions

Several harbour authorities and dredging companies were generously willing to donate freshly collected sediments in order to build a collection of North Sea coastal sediments. These samples varied greatly in grain size, salinity and metal pollution, as well as in function, ranging from industrial harbours to small-scale marinas. In addition, two sediments were chosen as reference sites as these were expected to be relatively pristine in terms of metal pollution, i.e. sediments from sandy, intertidal flats on the Wadden Islands Texel (The Netherlands, REF NL) and Sylt (Germany, REF G). The exact origin of the other 9 samples (NL, NL2, B1, B2, B3, G1, G2, G3 and G4) will not be exposed as requested by the involved authorities. All sediments were transported on ice to the laboratory, frozen and stored at -80°C until further use.

In the second part of this study one of the industrial harbour sediments, G1, was analyzed in greater detail, as part of a multidisciplinary research project focusing on Transport, REActions and Dynamics of heavy metals in marine sediments (acronym: TREAD), funded by the 5th framework directive of the European Union (EVK-CT-2002-00081) (sediment G1 is labelled HB in chapter 3). For this project, large-scale mesocosms (120x30x20 cm) were constructed and filled with sediments from a harbour basin in the North Western part of Germany. North Sea water (salinity 27-30‰) was permanently circulated through the mesocosms with a metal-free pump at flow rates between 9 and 10.5 cm s-1. In order to compensate for evaporation, de-ionized water was added when necessary. The mesocosms were kept at constant temperature (19°C) and illuminated 12 hours per day with fluorescent light. These sediments were analyzed regularly during one year, in terms of temperature, pH, salinity, oxygen and metal content, as described by Tankere-Muller and co-authors (2007). The mesocosm set-up allowed sediment cores to be taken of the upper surface and sliced at 0.5cm intervals, covering the upper four centimetres of sediment (8 samples). Samples were immediately frozen with liquid nitrogen, transported to the lab and stored in -80°C until further use.

DNA extraction

Total DNA was isolated in duplicates from sediment samples with the UltraClean Soil DNA extraction kit (MO BIO Laboratories Inc., CA, USA) according to the manufacturer's manual (treatment1). Approximately 0.25 g wet sediment was used as starting material. In order to assess the losses of DNA during the isolation procedure, a separate extraction was performed with the following minor modification to the protocol (treatment 2). Prior to the cell lysis step each sample was spiked with 10^7 copies of the empty plasmid pCR2.1-TOPO TA (3.9 kb) (Invitrogen, CA, USA). A Q-PCR recovery of 10^7 copies of pCR2.1 corresponded to 100% DNA recovery. The DNA recovery was used to calculate the copies of target gene to g of wet sediment. Total DNA quantification was performed for each sediment sample with PicoGreen (Molecular Probes, Inc., Eugene, OR, USA). As reference material, genomic DNA from pure cultures was isolated with the UltraClean Soil DNA protocol. Each procedure started with a 1.5-2 ml culture in the stationary phase. Cells were harvested and re-suspended in a small volume of supernatant (100-150µl). The rest of the extraction was performed according to manufacturer's instructions. Quality and quantification was determined with a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) The genomic DNA was stored at -20°C until further use.

Conventional PCR and Q-PCR

Primers were developed targeting the *cusA* gene in *S. oneidensis* MR1 and *Shewanella* MB4 (primer sequences 5' to 3', cusA_F: ATG ACG AAT GGC GTG AAG G and cusA-R: GAT ACG GGT TTT GAT GGG TTG, 112 basepairs PCR product). In addition, the *copA* gene in strain MR1 was amplified with the primers copA_F (CGC CAC CAT GAA CAA CAT GAA RCA RAA YYT, with Y = C or T) and copA_R (GTA ATG GAA GAC AGT GCC ATI GCI GC, with I = inosine, pairing with A, C or T), resulting in a product of 151 base pairs. These primers were tested on DNA from appropriate control strains (pure cultures) and

showed strong specificity. In addition, PCR products obtained from microbial community DNA extracted from metal-polluted harbour sediment were cloned in *E. coli* (TOPO TA cloning manual, Invitrogen, CA, USA). The high DNA sequence similarity between cloned PCR products confirmed the highly specific target gene amplification for both *copA* and *cusA* in the sediment DNA. For amplification of 16S rRNA genes, primers 1055F (ATG GCT GTC GTC AGC T) and 1392R (ACG GGC GGT GTG TAC) were applied as described elsewhere (Ferris et al., 1996).

Conventional PCR and Q-PCR were conducted as described previously (chapter 5). As far as the latter is concerned, briefly, amplification and detection of *cusA*, *copA*, spiked plasmids pCR2.1 and bacterial 16S rRNA genes were performed with the iCycler detection system (Bio-Rad Laboratories, Inc.). Reactions were performed in 25μl reactions (0.10-23ng DNA/reaction) with reaction components from the QuantiTect SYBR Green kit (QIAGEN GmbH). All PCR runs included sediment samples, no template controls, and standard DNA in 10-fold dilution series.

In order to asses DNA extraction efficiency and reproducibility, sediment samples spiked with pCR2.1 plasmid prior to extraction (obtained with treatment 2, see "DNA extraction"), were analyzed with Q-PCR using the M13F and M13R primers (GTA AAA CGA CGG CCA GT and CAG GAA ACA GCT ATG AC, respectively, 0.3 μM each). The reaction mixture further contained DNA template (0.30-9.0 ng/reaction), 0.08 μg/μl BSA and PCR grade water. Standard DNA was plasmid pCR2.1 without insert, concentrations from 100 to 10^7 copies/reaction. The PCR program started with 95°C for 15 min, followed by 45 cycles at 95°C for 1min and a combined annealing/extension step at 56°C for 30 sec.

After quantification, melting curve analysis was performed to confirm amplification specificity. The analysis was carried out in 80 cycles with 10 seconds per cycle, starting at 55°C and increasing 0.5°C per cycle. Detection limits and valid template DNA input ranges were determined by performing Q-PCR runs with dilution series of standards and sediment samples prior to quantification runs. Thresholds were set in the early exponential phase for each assay. Threshold cycle (CT) values (PCR cycle number at which the fluorescence emission of a sample exceeds that of no template controls) and the log starting quantities for standards in Q-PCR assays, were used to obtain standard curves from which target concentrations in sediment samples could be calculated.

Cloning, sequencing and phylogenetic analysis

During the research described here, cloning of metal resistance genes *cusA* and *copA* was done initially to test primer specificity on pure culture DNA and to provide molecular standards in Q-PCR. Secondly, the primers were tested on mixed culture DNA, extracted from metal-polluted sediment and the resulting products cloned into *E. coli*. Subsequently, 10 positive colonies were randomly selected from each clone library; the plasmids were isolated and inserts sequenced (20 total). The general cloning procedure was as follows: PCR fragments amplified from genomic or environmental DNA were extracted from 2% (w/v) agarose gels and purified with the QIAquick Gel Extraction kit (QIAGEN, Germany), cloned in pCR 2.1-TOPO TA plasmid and transformed into chemically competent TOP 10 *E. coli* cells, according to manufacturer's manual (Invitrogen, the Netherlands). Plasmids with inserts were extracted using the QIAprep Spin Miniprep kit (QIAGEN).

DNA sequencing was performed with the appropriate forward primers by BaseClear (Leiden, the Netherlands). Nucleotide sequences were compared to sequences stored in different nucleotide and genome databases. Selected sequences were used to generate alignments in clustalW (Lassmann & Sonnhammer, 2006), which were imported into Mega3 (Kumar et al., 2004). Final Neighbour-Joining trees were created with Kimura correction, for nucleotides, respectively.

Metal analysis

Metal analysis of the 11 sediments in the collection was obtained by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) measurements, kindly conducted by Dr Hao Zhang from the Aquatic Chemistry Research Group of Lancaster University (Lancaster, UK). Sample treatment was as follows: ~3g wet sediment was used in an extraction with 20 ml 0.01M HNO_3, shaken for 24 hours, filtrated through

0.45μm filter, and diluted 10 times for ICP-MS analysis in triplicate. Of the German harbour sediment, more information on metal concentrations and metal speciation was acquired during the course of the multidisciplinary TREAD project (Tankere-muller et al., 2007, also in chapter 3). In this study, work of Niko Finke (Max Planck Institute for Marine Micobiology, Bremen, Germany) is compiled into one depth profile of copper concentrations. Five separate cores were taken, sliced and treated as above, resulting in ICP-MS data for extracted copper in five separate replicates.

Results and discussion

Sediment characterisation and metal analysis

In the first part of this study, a sample collection consisting of 11 different sediments was subjected to the Q-PCR assay for detection and quantification of copper resistance genes copA and cusA in the microbial populations. Metal measurements were performed with ICP-MS analysis, results of which are shown in Table 1 for iron, manganese and copper, together with some basic sedimentary characteristics. Sediment particles of the seven industrial harbours listed in this table were of a very fine grain size and were classified as clay. Sediments from the two sampled recreational marinas (NL2 and G2) showed a combination of clay and sand, whereas the two reference sites (REF NL and REF G) consisted primarily of coarse sand grains. As most of the harbours and marinas were located along a river delta, and some more inland or in a confined basin, salinity was somewhat lower than average values in the North Sea (34-35‰), and therefore mostly defined as brackish. Iron varied between 6,3-94 mg/kg dry wt sediment, whilst manganese ranged from 0,26 to 186 mg/kg dry wt sediment. Copper analysis revealed concentrations of 7-110µg Cu/kg sediment dry wt. Highest values, i.e. above 40µg/kg, were found at the various sampled industrial harbours sites (G1, G3-4, B1-2, NL1). The selected reference sites were not as uncontaminated as presumed, since copper concentrations were moderately high (19-26 mg/kg sediment dry wt). The same was valid for other heavy metals as cadmium, cobalt and nickel, which were found in all sediments as well as the reference sites (data not shown).

Sensitivity and dynamic range of Q-PCR assay

In order to validate the Q-PCR assay, it was important to determine appropriate target concentrations. Initial experiments indicated that PCR amplification efficiency of molecular standards for copA and cusA was linear across 7 orders of magnitude, with minimal detectable concentrations of 10 copies/reaction (data not shown). This is in a slightly lower range than the 3 copies per reaction detected by Nyyssönen and co-authors, observed for linear detection of genes involved in naphthalene degradation.(2006). As the assay was to be conducted with DNA from environmental samples, and a number of PCR inhibiting compounds are known to be co-extracted during DNA isolation, further tests aimed to evaluate these effects and determine correct amounts of environmental input DNA.

As is shown in Figure 1, serial dilution of target DNA (logarithmic x-scale), obtained from a metal-polluted harbour sediment, resulted in a linear increase in threshold cycle (CT) values, for each of the four genes, i.e. copA, cusA, spiked plasmids and 16S rDNA. Dashed lines indicate the linear correlation between target DNA and CT values across a broad range of input DNA (0,1-23ng DNA), fitted with high probability.(R^2 values as follows: cusA, 0,978, copA, 0,974, spiked sediments, 0,963 and 16S rDNA, 0,985). Since the highest values of input DNA, presumably containing the highest amounts of co-extracted PCR-inhibiting compounds, did not deviate from the observed linearity, we can conclude that the quality of extracted DNA was good.

Table 1: General characteristics of 11 North Sea sediments and results of ICP-MS analysis for iron and manganese (in mg/kg sediment, dry weight) and copper (in µg/kg sed).

Sample	NL1	NL2	B1	B2	B3	G1	G2	G3	G4	REF NL	REF G
Function[1]	harbour	marina	harbour	harbour	harbour	harbour	marina	harbour	harbour	intertidal flat	intertidal flat
Grain type[2]	clay	clay/ sand	clay	clay	clay	clay	clay/ sand	clay	clay	sand	sand
Salinity[3]	brackish	marine	brackish	brackish	brackish	brackish	brackish	brackish	brackish	marine	marine
Fe (mg/kg)	14,58	7,75	12,41	8,91	8,38	14,64	7,81	15,57	12,30	6,26	94,49
sd (%)[4]	1,60	0,95	0,11	3,23	1,98	1,22	1,35	1,45	3,56	1,12	1,55
Mn (mg/kg)	93,04	0,26	30,39	22,02	18,88	126,83	61,15	185,72	133,68	0,74	1,46
sd (%)[4]	0,06	18,97	0,96	0,69	0,45	0,23	1,49	1,34	0,44	1,22	0,57
Cu (µg/kg)	43,85	7,01	49,73	65,56	29,53	103,17	15,50	55,14	42,19	19,00	26,13
sd (%)[4]	0,09	2,63	1,41	0,15	0,46	0,94	2,13	0,54	5,62	2,78	5,27

[1] Sites classified as harbour were of an industrial nature and had high amounts of cargo ships coming through on a daily basis. Sites classified as marina were small-scale harbours for the purpose of recreational yaughts. Two reference samples were collected from intertidal flats without any known history of polluting activities.

[2] Grain type determined by visual observation

[3] Salinity estimated upon location and local information; brackish defined as salinity between 0,5 and 30g/l.

[4] Standard deviation between triplicate analyses is shown as percentage

Primer specificity with microbial community DNA

While primer specificity had previously been tested with DNA of several control strains (chapter 5), in this chapter the tested target range was increased to DNA from an entire microbial community extracted from a metal-polluted harbour sediment (G1). By cloning the PCR products and creating a clone library for both *copA* and *cusA*, subsequent analysis of the inserts of 10 individual clones provided insight into the diversity of sequences targeted by the developed primers. Comparative sequence analysis amongst the clones and selected reference sequences led to the Neighbour-Joining trees shown in Figure 2A for *copA* and for *cusA* in 2B. The close phylogenetic relationship between the cop1-cop10 sequences and the cus1-cus10 sequences, suggests highly specific target amplification against the background of community DNA and other sediment components.

Pair-wise analysis of the 10 clones from the copA library resulted in an average 0,02 number of base differences per site and all sequences showed closest affiliation with *S. oneidensis* MR1. For *cusA*, differences between clones were even smaller (0,005 number of base substitutions), if existent at all. Close relatives of *Shewanella* strain MB4, originally isolated from the G1 sediment, appeared to be the major representatives of the *cusA* library. Despite care taken to incorporate degeneracies in the primers and therewith increase potential target range, our primers (containing up to 2 degeneracies) targeted only Shewanella like sequences in the tested sediment DNA.

Such high similarity between environmental clones was also observed after diversity analysis of *cadA* sequences in the cadmium-polluted Seine estuary (Oger et al, 2001, 2003). The cadA primers contained 6 degenerate bases and amplified sequences at least 98% similar to the original target organism *Staphylococcus aureus* (Oger et al., 2003)

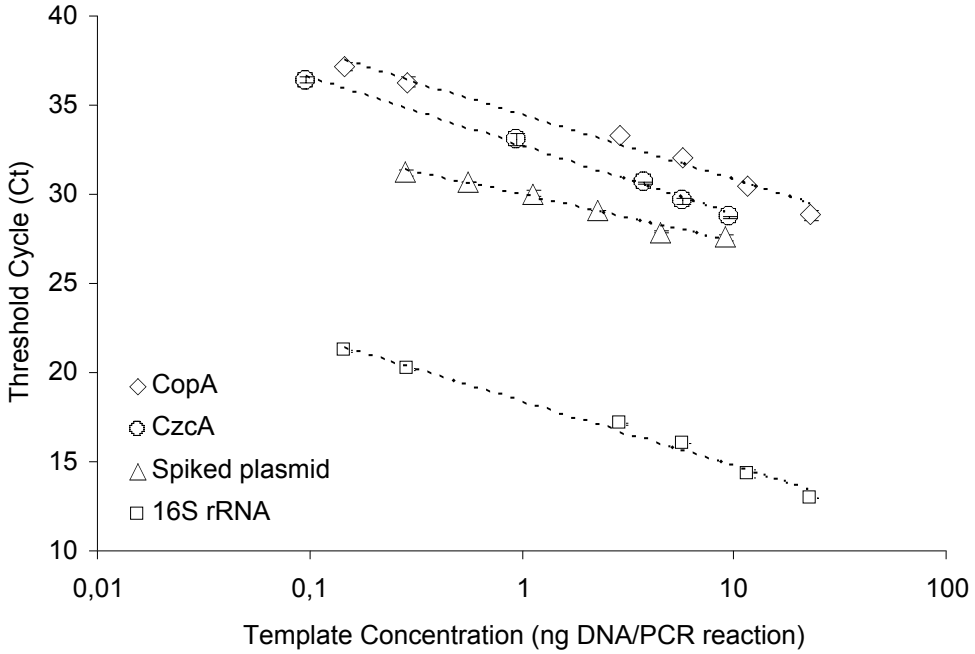


Figure 1: The effects of target DNA concentration and potential PCR inhibiting substances, co-extracted from sediments, on amplification efficiency in Q-PCR assays for four gene fragments: copA, cusA, spiked plasmids and 16S rDNA. Vertical error bars represent standard deviation between triplicate measurements. Linear regressions of logarithmic DNA concentrations versus threshold cycle (CT) values are shown with dashed lines; R2 values as follows: copA 0.974, czcA 0.978, spiked plasmids 0.963 and 16S rDNA 0.985.

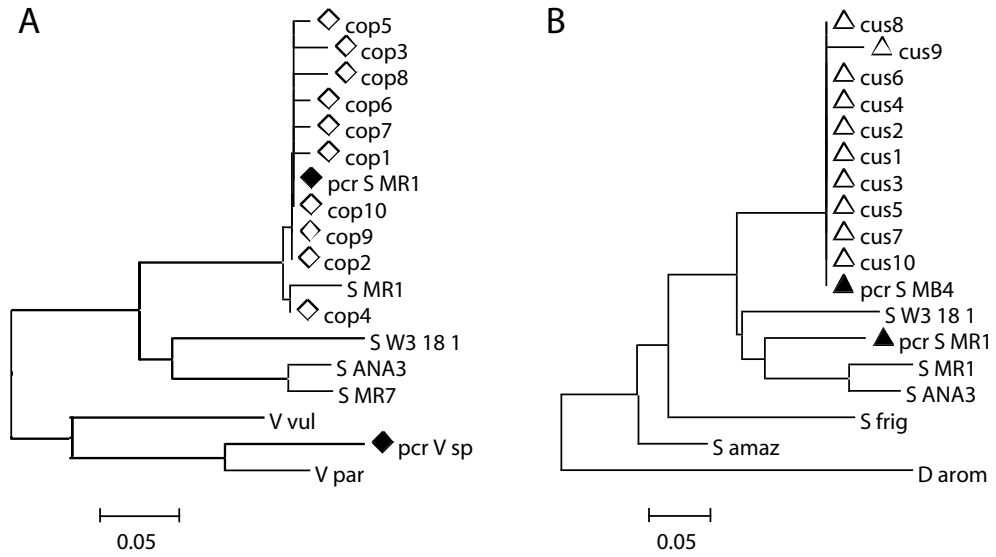


Figure 2: Neighbour Joining trees showing phylogenetic relationships between 10 nucleotide sequences of *copA* (A) and *cusA* (B), obtained by PCR amplification of microbial community DNA, extracted from a metal-polluted industrial harbour sediment, and subsequent cloning of PCR products. The cloned products are labelled cop1-cop10 (diamonds) and cus1-cus10 (triangles); sequences from PCR products obtained with pure cultures are also shown (filled symbols). Reference sequences used (genome accession numbers if available) and abbreviations as follows: S indicates *Shewanella*, V *Vibrio* and D *Dechloromonas*; S MR1, strain *S. oneidensis* MR1 (AE014299); strain W3-18-1 (CP000503), strain ANA-3 (CP000469), strain MR7 (CP000444), S frig, *S. frigidimarina* NCIMB400 (CP000447), S amaz, *S. amazonensis* SB2B (CP000507), S MB4, marine isolate MB4, V vul, *V. vulnificus* YJ106 (BA000037), V par, *V. parahaemolyticus* RIMD 2210633 (BA000031), V sp, marine *Vibrio* isolate and D arom, *D. aromatica* (CP000089). Scale bar represents 0.05 changes per nucleotide.

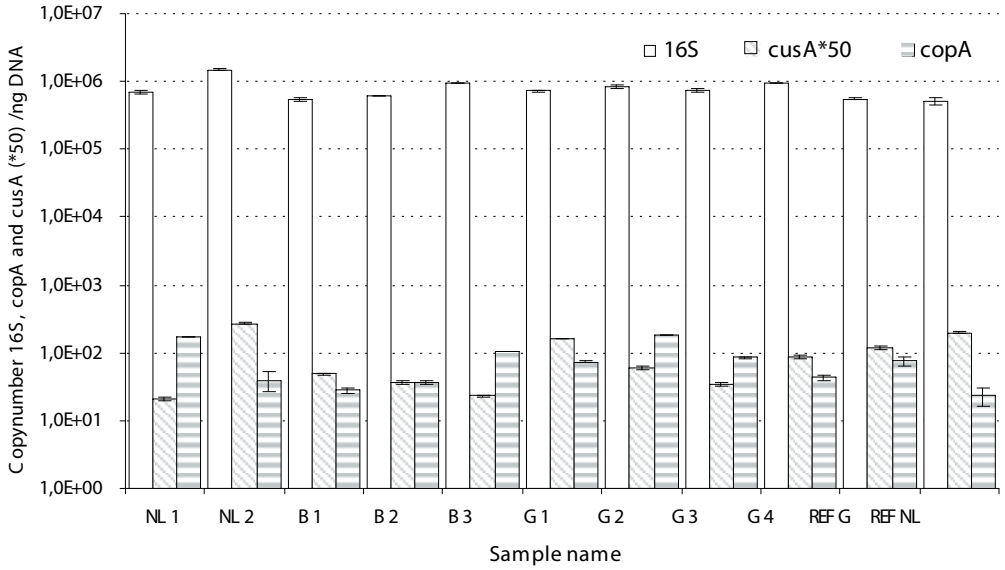


Figure 3: Quantification of copy numbers of 16S rDNA, *copA* and *cusA* genes in DNA extracts of microbial populations in 11 different North Sea sediments. Q-PCR results are shown on a logarithmic axis, expressed in copies per ng DNA (multiplied by 50 for cusA). Error bars represent standard deviation between triplicates.

Q-PCR on DNA extracted of 11 North Sea sediments

Microbial community DNA extracted from these sediments was used as target in Q-PCR analysis for analysis of *copA*, *cusA* and 16S rRNA genes. Figure 3 shows the copy numbers determined by Q-PCR of DNA samples from all sediments for the above mentioned three genes. The different bacterial populations were represented by equal numbers of 16S rDNA genes, around 10^6 copies/ng total DNA. Fragments of *cusA* and *copA* genes were detected in considerably lower numbers, i.e. up to 5 copies *cusA*/ng DNA and between 20 and 180 copies *copA*/ng DNA. In figure 4A the copy number of *copA* and *cusA* (per ng DNA) is depicted against the total extracted copper from the original sediment samples. The only significant correlation was found by splitting the sediment collection into two fractions, one clay (n=7), the other sand (n=4), containing the two reference sites (REF NL and REF G) as well as marinas NL2 and G2. The dashed line represents a linear fit between cusA (in clay fraction) and copper (R^2 = 0,78). Figure 4B provides an alternative normalization of *copA* and *cusA* copy numbers; dividing these by the amount of 16S rDNA copies detected, gives another representation of the number of metal resistance genes per bacterial population. The dashed line represents correlation between *cusA*/16S gene

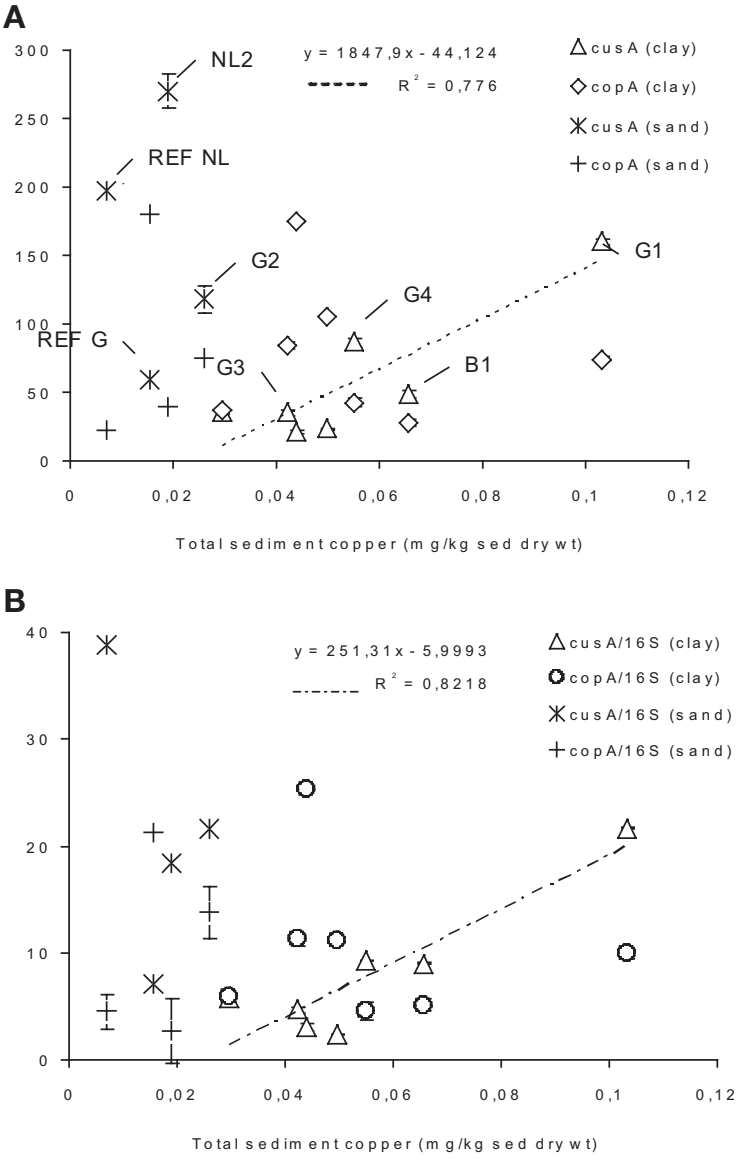


Figure 4: Correlation between copy numbers of *cusA* and *copA* and total extracted sediment copper of 11 North Sea sediments divided in fractions clay (n=7) and sand (n=4), standardised by total amount of DNA (A) or by total quantity 16S rDNA (B). Data labels are shown for some of the sediments in A. Error bars represent standard deviation between triplicate measurements; Dashed lines represent correlation between *cusA*/ng DNA and [copper] (A) and *cusA*/16S and [copper] (B), with the corresponding R^2 values of the fitted equation

copies and sediment copper, again limited to the fraction of clay sediments only (R^2= 0,82). For *copA* no significant correlation with copper was found, although detected numbers were at least 10-fold higher than *cusA* fragments.

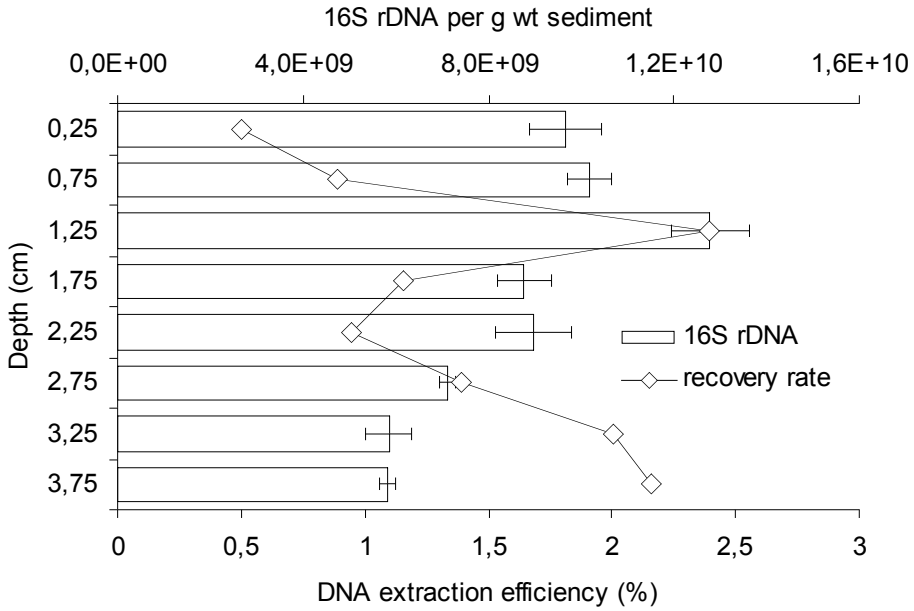
Considering that a matrix of fine clay particles has more potential binding sites for heavy metals compared to adsorption to sand grains, the apparent distribution of *cusA* and *copA* copy in two different fractions of sediments makes more sense; although total copper extracted from sandy sediments was in the low range (7-26μg/kg sediment), pore water concentrations may actually have been higher than concentrations in sediments with a clay texture (Warnken et al., 2004). This may explain the relatively high copy numbers of *copA* and *cusA* detected in sandy sediments REF NL, REF G, NL2 and G2. Regarding the clay fraction of 7 sediments, the most relevant data point was obtained from the sediment with the highest copper concentration, sampled from a major industrial harbour, G1. Besides high copper content, sediment G1 also contained high amounts of iron and manganese (Table 1). This is particularly relevant because manganese oxides were used as final electron acceptor for initial enrichments of both *S. oneidensis* MR1 and marine *Shewanella* MB4, the latter of which was originally isolated from G1 sediment. Although the distribution of *copA* copy numbers among the different clay fraction sediments was fairly even, the quantification of cusA genes suggested a prevalence in sediments with high manganese concentrations and located in the vicinity of G1 (e.g. G3 or G4). This may reflect differences in target specificity, as copA-primers also amplified sequences from a marine *Vibrio* isolate, or differences in target populations.

Q-PCR on DNA samples from a depth profile of G1 sediment

In the second part of this study, sediment G1 was investigated in more detail. Oxygen penetration depth varied between 2 and 8mm depth. Pore water analysis of heavy metals (described in chapter 3) showed that concentrations of both copper and cadmium peaked at, or just below, the sediment surface. These increased metal concentrations may be a result of aerobic degradation of metal-contaminated organic matter just below the surface. In order to investigate whether increased numbers of metal resistance genes *copA* and *cusA* would coincide with the observed subsurface peak of dissolved copper, two replicate sediment cores were taken and the top 40mm carefully sliced at 5mm intervals.

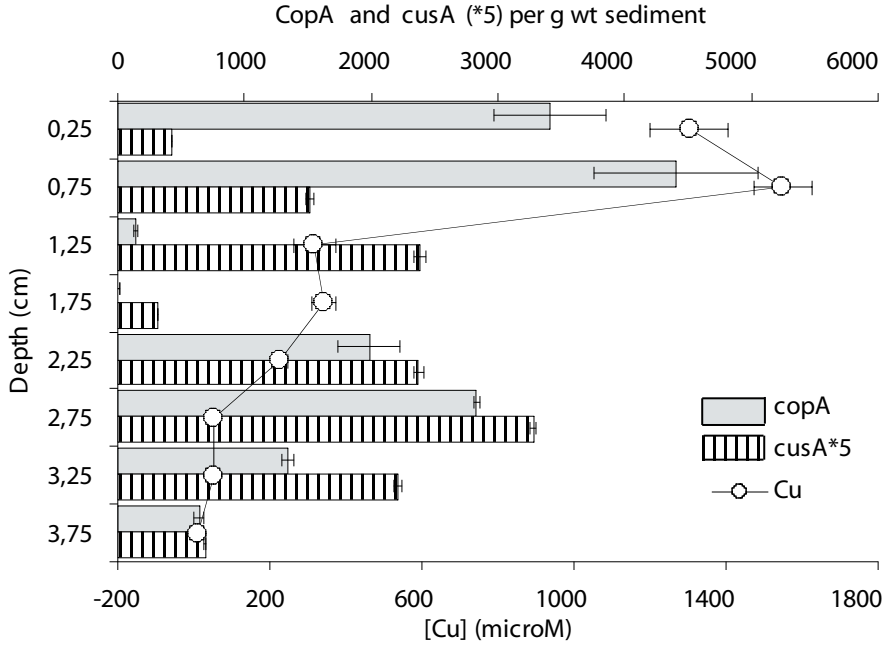
In order to estimate the reproducibility and efficiency of the DNA extraction procedure, known amounts of plasmids were added to the sediment samples prior to extraction (see treatment 2 in material and methods). Complete retrieval of all added plasmids would indicate a 100% recovery rate. Figure 5 shows the 16S rDNA gene copy number and the % of recovered plasmid DNA of two replicate cores along a 4cm depth gradient. The copy number of 16S rDNA genes measured fell within a relatively narrow range, between $6x10^9$ and $1x10^{10}$ per g of wet sediment, generally declining with depth. The recovery rate of spiked plasmids was fairly low, average 1.43±0.32 (%), but well above detection limit and, more importantly, similar among the different samples tested. Dionisi and co-authors showed that 98% of spiked lux genes could be retrieved after soil DNA extraction (1994). This high number may be explained by the relatively high amount of spiked material used (10^9 copies instead of 10^2 copies) and the fact that no extensive mechanical cell disruption was applied, in order to prevent shearing of DNA molecules (whereas a 10 minute bead-beating step was included in out protocol), The value of 2% should therefore not be regarded as an actual extraction coefficient; addition of whole cells instead of plasmid DNA would have been preferable in that case. However, as similar recoveries of different sediment samples were obtained, the main goal of the experiment was met by showing that extraction of sediment DNA was a reproducible procedure.

The copy number of *copA* and *cusA* genes per g of wet sediment along a 4cm depth gradient is depicted in Figure 6, together with the average copper concentration (in μM) measured in 5 replicate cores. The top cm of sediment displays the highest copper concentrations of around 1,5mM and decreases with depth. This peak in copper concentrations coincided with maximal numbers of *copA* fragments detected, approximately 3500 copies per g of sediment, declining with further depth. For *cusA* maximal values were detected at a depth of 3cm, approximately 660 copies *cusA* per g of sediment. For neither of the copper resistance genes a significant correlation was found with the typical subsurface copper peak, yet depth and gradients did seem to have a determining factor in the distribution of *copA* and *cusA*. Accordingly, these fluctuations in copy numbers may reflect the presence of *Shewanella* involved in the aerobic and anaerobic lifestyle.



^ Figure 5: Duplicate sediment cores of harbour site G1 sampled along a depth gradient with 5mm intervals, were analyzed with Q-PCR for quantification of 16S rDNA genes per g of wet sediment (top axis) and spiked plasmids (added prior to DNA extraction, expressed as extraction efficiency in %, bottom axis). A depth of 0cm indicates the sediment water interface. Error bars represent standard deviation between biological duplicates analysed in triplicate.

v Figure 6: Duplicate sediment cores of harbour site G1 sampled along a depth gradient with 0,5cm intervals, were analyzed with Q-PCR for quantification of *copA* and *cusA* (*5) genes per g of wet sediment (top axis) and extracted copper concentration (in µM, average of 5 replicate cores, standard deviation shown, bottom axis). A depth of 0cm indicates the water sediment interface. Error bars represent standard deviation between biological duplicates analysed in triplicate.



Conclusions and recommendations

It seems probable that in case of heavy metal pollution, growth of more metal-resistant types of micro-organisms is stimulated, whereas metal-sensitive species disappear (or acquire the appropriate gene(s) and survive). Therefore, it can be hypothesised that microbial populations in metal-polluted sediments posses a higher number of metal resistance genes compared to pristine sediments, reflecting this increased level of metal-adaptation. Although this research provides some basic explorations, enabled by the Q-PCR detection of *copA* and *cusA* in marine sediments, the specificity of the primers limit the target population; therefore the distribution of these metal resistance genes could simply reflect the presence of *Shewanella* or other representatives of the γ-subclass of the Proteobacteria. Primers with a broader species specificity might be useful, especially in the case of comparing different sediments. Another complicating factor in the application of molecular techniques, is the potential bias of the selected DNA extraction method with regard to certain types of Bacteria or sediments (Frostegard et al., 1999). Although the first step in many commonly applied protocols, detailed investigations of sediment extraction efficiency are not often published (Oger et al., 2001; Dionisi et al.,2004). For future recommendations, the extraction of mRNA of sediments and subsequent Q-PCR analysis might reveal a more accurate image of the number of metal resistance genes in active use.

Acknowledgements

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Sampling at the intertidal flat finished just before high tide

7 Discussion and outlook

Heterogeneity of sediments and reproducibility of molecular methods

Water bodies, sediments and submerged soils often show a variety of geochemical gradients, which can be exploited by many Bacteria and Archaea. The oxidation of organic matter is coupled to reduction of various inorganic electron acceptors, depending on their electron potential and availability. Our research focussed on the role and toxicity of (heavy) metals and toxicity in the microbial community of marine sediments. As sediments are known to be very heterogeneous a central question in the first part of this research was to estimate the reproducibility of molecular methods on sediment samples. A related objective was to determine how many samples needed to be analyzed, in order to provide a reliable view of microbial diversity. Reproducibility of 3 individual DNA extractions and subsequent PCR-DGGE was high, when applied on fine-grained sediments with high clay content, but appreciably lower in sediment with a coarse sand grain. This might lie within the line of expectation, as sediments with high clay content represent relatively impermeable systems and are assumed to be diffusion-controlled. Contrastingly, due to the high permeability of sandy sediments, coarser grained systems are defined as convection controlled. This implies that sediment structure is determined by currents through the sediment, which may result in frequent flushing of one site whereas an adjacent patch is not reached by the flow of water. Indeed, the sandy sediment appeared to be heterogeneous to such an extent, that significant differences in microbial population were sometimes observed within and between mesocosms. However, differences in the recorded DGGE profiles were generally small and reproducibility of the applied method was considered sufficient to continue experiments.

Different methods to extract genomic DNA from environmental samples as soils and sediments have been tested and published (Steffan et al., 1988; Zhou et al., 1996; Bürgmann et al., 2001). Our results indicated that sandy sediments repeatedly yielded lower DNA concentrations, possibly due to lower amounts of microbial biomass in these sediments. Zhou and co-authors stated that DNA yield was positively correlated with organic carbon content in soils (1995), confirming our findings of high DNA content in silty sediments rich in organic matter. As has also been stated by Bürgmann and co-authors (2001), it is practically impossible to develop one DNA extraction protocol that will give optimal results for each type of soil or sediment. In order to truly evaluate DNA extraction efficiencies between different sediments, one would have to spike samples with an internal standard before DNA extraction. Such experiments were performed with different depth samples of HB sediment, and showed reproducible DNA extraction efficiencies, independent of depth.

Spatial and temporal variation during mesocosm incubation

In order to enable frequent and spatially accurate sampling, and to limit the number of uncontrollable environmental factors, the experimental design was based on the use of mesocosms, glass incubation chambers containing submerged sediment samples with controlled water flow, pH, light, etc. Mesocosms of varying size and complexity have been used previously to study microbial communities under ambient conditions (Frischer et al., 2000; Riemann et al., 2000), and have also proven valuable in contamination studies (Schwinghamer, 1988; Arias et al., 2003; Hendrickx et al., 2005). However, in order to validate mesocosms as models for natural ecosystems, reproducibility assessments were required. Despite the widespread use of mesocosms, this subject has so far received little attention in literature

(Roeselers et al., 2006). Our results with large-scale mesocosms, containing relatively pristine intertidal sediment (IF) and polluted harbour (HB) sediment, indicate that spatially separated, individually treated, sediment samples may result in very diverse profiles of microbial communities. Yet mixing of individual DNA preparations before PCR amplification, generally improved the total number of species represented in the DGGE profile.

Vertical depth profiles of archaeal and bacterial communities in HB sediment showed little variability at 1cm and 0.5cm resolution. Primary changes in PCR-DGGE profiles of microbial populations occurred at around 1cm depth, coinciding with the average oxygen decline at approximately 8mm, and therefore possibly indicative of a separation between oxic and anaerobic communities. Finally, comparison between DGGE profiles of the freshly collected sediments (jun 03) and DGGE patterns of the sediments, after incubation in the mesocosm for one year (oct 03-oct 04), revealed a remarkably stable community composition for Archaea, Bacteria and Cyanobacteria in the HB sediment. Microbial populations in the sandy IF sediment, showed temporal changes in both the disturbed and the control mesocosm. The exact driving forces behind these shifts in dominant bacteria remain unknown, but it seems reasonable to assume that adjustment to mesocosm conditions plays a role in the apparent succession. Applied mesocosm conditions were after all more similar to a sediment on the bottom of a harbour basin, than to the high current environment provided by an intertidal flat. The use of 16S rRNA clone libraries, generated more data on different species present in a microbial community, and with higher resolution than obtained with DGGE. Unfortunately however, the high amount of work involved in analyzing these libraries, makes this approach less suitable for a reproducibility study in this context.

Observed microbial diversity in marine sediments

By combining phylogenetic information retrieved from DGGE profiles and bacterial 16S rRNA clone libraries, a global overview of microbial diversity in sediments HB and IF evolved. DGGE analysis of samples from depth profiles of HB sediment (2mm interval) allowed for a detailed characterization of variations in the microbial population with depth. In the upper sediment layers (0-1cm), opportunistic γ-Proteobacteria were detected as *Pseudoalteromonas, Photobacterium* and *Halomonas*. These species are typical for surface layers of sediment, where so-called r-strategists can proliferate because they have the first choice of nutrients coming in from above. In lower regions (below 1cm), sequences affiliated to (facultative) anaerobes as *Shewanella* were found. These results are in accordance with Grundmann's findings (2004), who described high small-scale diversity at millimetre scale in soil systems and generally agrees with the steep gradients expected in fine-grained sediments.

Analyses of clone libraries showed that most bacterial species detected belonged either to the *Bacteroidetes* or the Proteobacteria (the α- and γ-subclasses). For sediment IF the division between those two major groups was about fifty-fifty, as seems to be typical for a number of marine environments such as the North Sea, the Mediterranean Sea, Arctic and Antarctic ice, psychrophilic sediments and marine snow assemblages (Bowman et al., 1997; Eilers et al., 2000; Brinkmeyer et al., 2003; Bowman & McCuaig, 2003). Molecular analysis of the bacterial community in HB sediment further revealed a number of species with sequences affiliated to the phyla *Haloanaerobiales* and the *Acidobacteria*, clustering with sequences in candidate divisions JS1 and OP8. About one third of each library consisted of sequences that were most closely related to uncultured micro-organisms according to similarity analysis with sequences stored in major databases.

One of major differences between the outcome of DGGE and clone libraries, was the high abundance of species belonging to the Bacteroidetes, detected with the latter method. This may imply that the primers used for Bacterial PCR-DGGE have less affinity for target sequences of this group. Members of the *Bacteroidetes* are characterised as decomposers of dead biomass, degrading primarily (complex) polymers. Marine *Cytophaga* species are (facultative) aerobes, known to attack chitin, pectin, keratin and even agar. The marine clade appears to have ecological impacts beyond simple mineralisation-based commensalisms. For instance, *Cellulophaga* and *Psychroserpens* are both capable of actively attacking and killing various algal species, in particular dinoflagellates, by producing extracellular enzymes and toxic compounds (Bernardet & Nakagawa, 1995). The lysis of algal cells in blooms causes a rapid local increase in dissolved and particulate organic matter which supports bacterial growth. In this respect, toxic

secondary metabolites allow bacteria to play an aggressive, predatory role in food webs.

Nearly all retrieved sequences from the Archaea were affiliated to the *Euryarchaeota*, with closest matches to other environmental sequences originating from sediments of the Baltic Sea, the Black sea and the Sea of Japan. However, one of the dominant bands in the DGGE profile of the sandy sediment belonged to the *Crenarcheota*, and was related to the recently isolated ammonia-oxidizing *Nitrosopumilus maritimus* (Konneke et al., 2005). The phototrophic community in harbour sediments was shown to be relatively stable in time and dominated by cyanobacteria, with major representative species affiliated to *Synechococcus*. In contrast, analysis of the sandy sediments showed the prevalence of diatoms, closely related to chloroplast rRNA of *Amphora delicatissima*. Successive changes were observed in the composition of the photosynthetic community in sandy sediments. These changes may be due to bioturbation, grazing pressure, or the shift from natural to artificial light conditions.

Distribution of heavy metals in marine sediments

Pore water heavy metal content in HB sediment was higher than in IF sediment, but similar to maximum concentrations observed in other sites throughout the North Sea and river sediments, with the exception of copper (BSH report, 2002). Regarding this particular metal, Shiff and co-authors (2004) quantified the emission from standard epoxy coatings on a typical recreational vessel to be 4μg Cu cm^{-2} day^{-1}. A different study in Queensland, Australia (Warnken et al., 2004), indicated that at sites where more than 30 ships pass per day, the copper concentrations in most cases exceeded the relevant environmental guideline (1.3μg l-1). In comparison, pore water copper concentrations detected in mesocosm HB were at least 50-fold higher, and 10- to 20-fold elevated, when compared to values in other North Sea sediments. This copper pollution is primarily connected to the fact that the harbour basin is located in the vicinity of an industrial wharf.

Deposition of a 3 mm layer of metal-polluted HB sediment on a sandy sediment caused an increase in pore water copper concentrations of surface sediments (4-fold compared to sand control and 1.5-fold compared to harbour control). This effect was of a transient nature and had mostly disappeared after one year of mesocosm incubation. Elevated pore water metal concentrations as a result of re-deposition of polluted sediment were also described in other studies (Leipe et al., 2005). However, the degree of impact depends on the volume of deposited material and its contamination level, as Chen and co-authors (2003) only observed an increase in copper levels after deposition of a 5cm layer of polluted sediment, while no effects could be detected underneath a 1cm layer.

In the fine-grained HB sediment, profiles of pore water copper and cadmium concentrations peak at the surface or just below. High surface concentrations were probably caused by the microbial oxidation of metal-contaminated organics at the sediment surface (Tankere-Muller et al., 2007). Since oxygen penetration depth varied between different time points, release of copper and cadmium in the subsurface (below 1cm) could also be caused by the anaerobic reduction of heavy metal-containing iron(hydr)oxides (Markwiese and Colberg, 2000). After three months of bio-turbation with Nereis diversicolor iron, copper and cadmium pore water concentrations were lowered approximately 10-fold at all sediment depths, due to mixing by the polychaete worms and subsequent chemical oxidation, adsorption or precipitation.

Changes in diversity and metal-tolerance after metal exposure

Changes in bacterial diversity as a result of the deposition of metal-polluted sediment were not readily observed in the DGGE profiles. Regarding the archaeal population, a slight decrease in diversity was detected, directly following sediment deposition. After one year of mesocosm incubation, however, similarity between DGGE profiles of disturbed and control communities increased again. This susceptibility of Archaea to heavy metals was also noted by Sandaa and co-workers (1999), while investigating shifts in community composition in soils amended with heavy metals.

Comparative analysis between clone libraries of HB and IF sediment showed several groups of highly similar, but not identical clones. These “shared” clusters were most closely related to *Ruegeria atlantica*, *Rhodobacter sphaeroides, Vibrio splendidus* and a group related to environmental clone BrownBay 2-

71, within the *Flavobacteriaceae*. Interestingly, clone BrownBay 2-71 actually originated from a heavy metal-polluted Antarctic sediment (Powell et al., 2003). With the applied experimental set-up, it was unfortunately not possible to determine whether these species were indigenous to both sediments, or actually transferred from HB to IF by sediment deposition. Toxicity tests with collections of isolates however, underlined the effects of the deposition of metal-polluted sediment by showing a substantial increase in copper- and cadmium-tolerant aerobic heterotrophs, compared to results for the harbour control and sand control. Elevated levels of metal-tolerant Bacteria in soils and sediments after exposure to heavy metals have been described previously (Diaz-Ravina and Baath, 1996; Ramaiah and De, 2003, Rasmussen et al., 1998), but not in combination with detailed metal analyses indicating such localized and short-term metal exposure. It seems unlikely that this adaptation concerns numerically dominant micro-organisms, as large community shifts were not observed in DGGE profiles, except, initially for the archaeal population. It remains to be determined whether the increase in metal-resistance is due to the proliferation of metal-tolerant bacteria, originating from the deposited metal-polluted sediment, or whether horizontal gene transfer of metal-resistance genes may have played a role.

Metal-tolerance of metal-reducing *Shewanella* isolates

Considering that Fe(III)- and Mn(IV)-oxides may represent relevant sinks for heavy metals in marine sediments, sediment samples from IF and HB were used as inoculum for enrichment of marine metal-reducing bacteria. Three isolates were obtained. Through serial dilution of cultures with δ-MnO_2 as final electron acceptor, strain MB4 was obtained from HB sediment (99% similarity to 16S rRNA gene *S. marisflavi*). 16S rRNA sequences similar to isolate MB4 were detected previously in DGGE profiles of the original sediment samples and as minor constituents of the bacterial population in sediment HB according to clone library data. This suggests that isolate MB4 represented numerically relevant populations of Bacteria, confirming the wide-spread occurrence of members of the genus *Shewanella* in aquatic habitats (Ziemke et al., 1998; Ivanova et al., 2003), as well as the fact that they belong to the readily culturable bacteria. Offering Fe(III) as electron acceptor led to isolation of strain FB18 (98% similarity to *S. colwelliana*) from the harbour sediment. From the sandy sediment, a very similar Fe(III)-reducing bacterium was isolated, strain FS8 (97% similarity to *S. colwelliana*).

Maximal metal concentrations (Co, Zn, Cd and Cu) at which growth was observed with isolates MB4, FB18, FS8 and type-strain *S. oneidensis* MR1 presented in this paper were in the micromolar range under all tested conditions, in contrast to values published for *E. coli* and *C. metallidurans*, which are in the millimolar range (Nies, 1999). The range of tolerated metal concentrations values determined here for MB4 and FB18 did, however, correspond to metal concentrations measured in the original harbour sediment. Of the four tested strains, isolate MB4 was most tolerant to cadmium and copper under aerobic conditions. On the other hand, isolates FB18 and FS8 were more resistant to cobalt and zinc, when compared to strain MR1 and MB4. Toxicity effects were evident from increased lag-phases, reduced growth-rates or a combination of both. Under anaerobic conditions with fumarate as electron acceptor, tolerance for copper remained more or less unchanged in strains MR1 and MB4. When fumarate was exchanged by Fe(III)- or Mn(IV)-oxide as electron acceptor, an increased tolerance level was observed in MR1 and MB4, presumably by a decrease in bio-availability of copper.

Bio-availability determines toxicity of heavy metals

Speciation and bio-availability are keywords in the relation between total metal content of a system and the resulting effects for biota. Besides heavy metal immobilisation in marine sediments as metal sulphides, numerous other potential metal-binding types and sites exist among different particles of a sediment matrix (Naylor et al., 2006). As metal-ion binding is pH-dependent, it is difficult to reliably quantify the fractions of available heavy metal (Plette et al., 1999). Naturally, the same observations hold for experimental conditions during pure cultures studies. Campbell and co-authors demonstrated that the amounts of Cd, Zn and Cu sensed by a genetically modified strain of *E. coli* were heavily influenced by concentrations of EDTA and fulvic acid (2000). A different study focusing on chromate reduction by *S. oneidensis* under different growth conditions, showed that in medium containing 20mM of lactate, only 13-14% of the reduced Cr(III) remained soluble, presumably as a complex to lactate of components of

yeast extract or peptone (Middleton et al., 2003). When 100mM of HEPES was included as buffer, the percentage soluble Cr increased to 47%. An important difference between heavy metal-reduction and -resistance is, however, the fact that an electron acceptor may be utilized outside the cell, whereas presence of a heavy metal inside the cell is required for toxicity effects. Our results regarding copper availability in different media with *Shewanella* isolate MB4 and type-strain MR1, proved that toxicity was largely determined by the amount of yeast extract or peptone supplemented; although substantial growth was observed in presence of 750μM of copper in 0.6-fold diluted LB broth, a minimal medium only supported growth at copper concentrations below 75μM. Apparently, these media components complexated copper in such a way that they were no longer toxic to *Shewanella*.

A bi-phasic experimental design with δ-MnO_2-reduction followed by use of fumarate, furthermore indicated that the presence of manganese oxides decreased bio-availability of copper through sorption processes, thereby alleviating the toxicity of copper to strain MB4 to some extent. By offering δ-MnO_2, with copper at concentrations around maximal adsorption capacity as final electron acceptor, we showed that the onset of copper toxicity in isolate MB4 was delayed until all δ-MnO_2 had been reduced. Challenging MB4 with δ-MnO_2 incubated in copper concentrations 25-fold higher than maximal adsorption capacity, resulted in immediate toxicity during Mn(IV)-reduction. Scanning electron microscopic images showed the initial amorphous Mn(IV)-oxides and newly formed, highly crystalline, lemon-shaped, particles making up the precipitate that remained after microbial reduction. Concomitant electron dispersive x-ray spectrometry confirmed presence of copper in the initial sample, yet detected no copper in the precipitate after microbial reduction, indicating that the Mn(IV)-reducing *Shewanella* strain MB4 immobilized copper adsorbed to δ-MnO_2.

To the best of our knowledge, this is the first report of copper mobilization by a Mn(IV)-reducing bacterium. Although Markwiese and Colberg demonstrated copper release from an ironhydroxide phase, they used a mixed microbial consortium containing fermentative bacteria and Fe(III)-reducers (2003). Their findings suggested that an increase of Cu in the culture medium significantly extended the time before Fe(III) reduction occurred and decreased the reduction rate, but did not affect the extent of hydrous ferric oxide (HFO) reduction, which is largely in agreement with our observations.

Quantification of *copA* and *cusA* in *Shewanella* mRNA and sediment DNA

The development of a Q-PCR assay for detection of copper determinants copA (an ATPase type transporter) and cusA (a metal/proton antiporter), enabled monitoring of gene expression in *Shewanella*. Under aerobic growth conditions, the correlation between the presence of copper (25μM) and expression of presumed copper determinant *cusA* was significant in both MB4 and MR1 ($p \leq 0.0006$). Further aerobic tests pointed out that *cusA* expression in MB4 increased approximately 2-fold between 25 and 100μM of copper, and was also significantly induced by cadmium ($p=0.0013$). *CusA* was detectable in MR1 under all conditions tested and copy-numbers were significantly higher in cultures grown in presence of either copper or cadmium (all $p<0.0001$). When anaerobic growth conditions were applied and fumarate offered as terminal electron acceptor, *cusA* expression in presence of 100μM of copper was lower than under aerobic conditions, but still significantly higher than in controls (MB4: $p=0.038$, MR1: $p=0.0059$). Interestingly, *copA* was only significantly induced under anaerobic conditions ($p<0.001$) in MR1. These findings are in contrast with results obtained for *E. coli*, which indicated that the copA protein was primarily induced under aerobic conditions, whereas cusA was essential for full anaerobic copper tolerance (Outten et al., 2001; Kershaw & Brown; 2005). However, the operon encoding *copA* in *E. coli* also encodes *cueO* , a periplasmic oxygen-dependent oxidase. Perhaps, the absence of an oxygen-dependent multicopperoxidase such as *cueO* in *S. oneidensis* MR1 may help explain why copper resistance does not markedly change when comparing aerobic and anaerobic conditions.

Involvement of *copA* and *cusA* in copper homeostasis of *Shewanella* was expected, based on homology with related proteins and the presence of several signature amino-acid motifs (Paulsen et al., 1996; Rensing et al., 2000; Fan et al., 2002; Coombs and Barkay, 2005). These assumptions were confirmed by the above described expression analysis. In order to determine whether quantification of *copA* and/or *cusA* in sediment DNA might also be correlated to bio-available copper in marine sediments, final experiments concentrated on Q-PCR analysis of sediment DNA. Initially, 11 different North Sea sediments

were subjected to the Q-PCR assay for the copper resistance genes in the microbial populations. The only significant correlation between copper and sediment *cusA* (R^2 = 0,78), was found by splitting the sediment collection into two fractions, one clay (n=7), the other sand (n=4). No evident pattern for *copA* distribution was detected.

In order to investigate whether the subsurface peak of dissolved copper measured in sediment HB, would coincide with increased numbers of *copA* and *cusA* genes, replicate sediment cores were taken and the top 40mm carefully sliced at 5mm intervals for concomitant metal and DNA analysis. Although maximal copper concentrations coincided with maximal numbers of *copA* fragments, i.e. approximately 3500 copies per g of sediment, less dependency was shown in lower regions of the sediment. For *cusA* maximal values were detected at a depth of 3cm, approximately 660 copies *cusA* per g of sediment. Although we could not identify a significant correlation between the copper resistance genes and total or pore-water copper concentrations, vertical gradients did seem to have a determining factor in the distribution of *copA* and *cusA*. In the future a higher resolution down to the mm scale should be investigated to further test the expected relation between available toxic metals and metal resistance.

Potential applications of metal-resistant bacteria

Fundamental research of metal-resistance revealed some of the underlying mechanisms of metal detoxification in micro-organisms and elucidated the complexity involved in metal homeostasis. The importance of studying these subjects is underlined by the fact that impaired copper homeostasis causes fatal disorders in humans, such as Menkes' disease and Wilson's disease; The discovery that mutations in the human ATP7A gene, a homologue of bacterial *copA*, hamper copper uptake by cells, has made treatment with chelators possible (Rensing et al., 2000). In addition, metal-resistance is often linked to virulence, for instance, in *H. pylori* a RND protein conferring resistance to cadmium, zinc and nickel is essential for colonization of the gastrointestinal track (Stahler et al., 2006). On the subject of multiple drug resistant bacteria, there is also growing concern that metal contamination functions as a selective agent in the proliferation of antibiotic resistance, as genes encoding these functions are often on the same genetic element such as a plasmid, transposon or integron (Baker-Austin et al., 2006).

Metal-resistant bacteria can be applied in industrial processes to mobilize, concentrate or purify heavy metals; e.g. *Pseudomonas* sp. strains can be used in biosorption processes (Leung et al., 2000), sulphur-oxidizers as *Thiobacilli* are employed to leach heavy metals from ores (Suzuki, 2000; Rawlings, 2001, 2002; Rawlings et al., 2003), sediments (Chen and Lin, 2000) and sewage sludge (Shanableh & Ginige, 2000), biofilms of sulphate-reducing *Desulfobacteriaceae* can precipitate metal sulphides (Kuenen et al., 2000, Labrenz et al., 2000) and remediate acid mining wastes (Gibert et al., 2002), and an arsenate-resistant thermophilic consortium has proved very capable of purifying gold from arsenopyrite (Hallberg et al., 1996). The metal-resistant *C. metallidurans* was genetically modified to incorporate mouse metallothioneins on its cell surface and was shown to ameliorate cadmium toxicity for plants in soil systems by metal sorption (Lovley and Lloyd, 2000). In another study, this bacterium was equipped with a mer-lux fusion gene, creating a biosensor for Hg^{2+}, a tool useful to measure metal bio-availability (Nies, 2000; Bontidean et al., 2000). When metal-resistant bacteria additionally posses metabolic pathways for degradation of xenobiotics, they can be applied in remediation of polluted soils or aquifers with high background levels of metals.

As heavy metals in marine ecosystems may be correlated with sinks of iron- and manganese oxides, bacteria reducing these substances could play a role in mobilization of heavy metals (Markwiese and Colberg, 2000). Manganese oxides are such excellent scavengers of heavy metals (Takematsu, 1979; Lewis and Landing, 1992; Tebo et al, 2004; Naylor et al, 2006), that they are even used as a filter to remove heavy metals from drinking water (US patent 5082570, 1992). Metal-reducing metal-resistant bacteria may prove valuable in solubilising heavy metals from these types of matrices under controlled conditions in wastewater systems.

Outlook

It seems probable that in case of heavy metal pollution, growth of more metal-resistant types of micro-organisms is stimulated, whereas metal-sensitive species disappear (or acquire the appropriate gene(s) and survive). To actually demonstrate this phenomenon in a laboratory setting has proven very difficult. Both molecular methods and cultivation-based approaches are accompanied by their potential biases. The heterogeneity of the sediment structure creates an even greater challenge. Although the research described in this thesis covers a rather wide range of related topics, at least some more insight is gained in metal-microbe interactions in marine sediments. Few of the initial the questions can now be answered, and even more new questions raised. For future recommendations, the extraction of mRNA of sediments and subsequent Q-PCR analysis might reveal a more accurate image of the number of metal resistance genes in active use. The development of primers targeting a wider range of species or proteins can add to existing knowledge of environmental gene pools available and dispersion of resistance genes. Additionally, knock-out mutants may provide more specific information on the interplay between *copA* and *cusA* in copper homeostasis in *Shewanella*.

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Sampling sediments from a dredging ship

Personalia

Ann-Charlotte Margareta Toes was born on September 30th 1979 in Schiedam, the Netherlands. After completing her secondary education at the Baarnsch Lyceum in Baarn, she enrolled in the study of Biotechnology at Wageningen University in 1997. She specialised in Molecular Biology and Environmental Microbiology with several graduation projects. Initially, she studied the microbial ecology and physiology of waste water treatment systems at the department of Microbiology in Wageningen. Subsequently, she joined a research project concerning the microbial breakdown of polyaromatic compounds at Lund University in Lund, Sweden. At the company BioClear in Groningen, the Netherlands, she investigated microbially influenced corrosion. After finishing her MSc. degree (Ir.) in 2003, she started as a PhD student at the department of Biotechnology at Delft University of technology. In her doctoral research she was supervised by prof. dr. J Gijs Kuenen and dr. Gerard Muyzer. Her research focused on the effects of heavy metals on microbial diversity and metal resistance and was part of a multi-disciplinary European Union project.

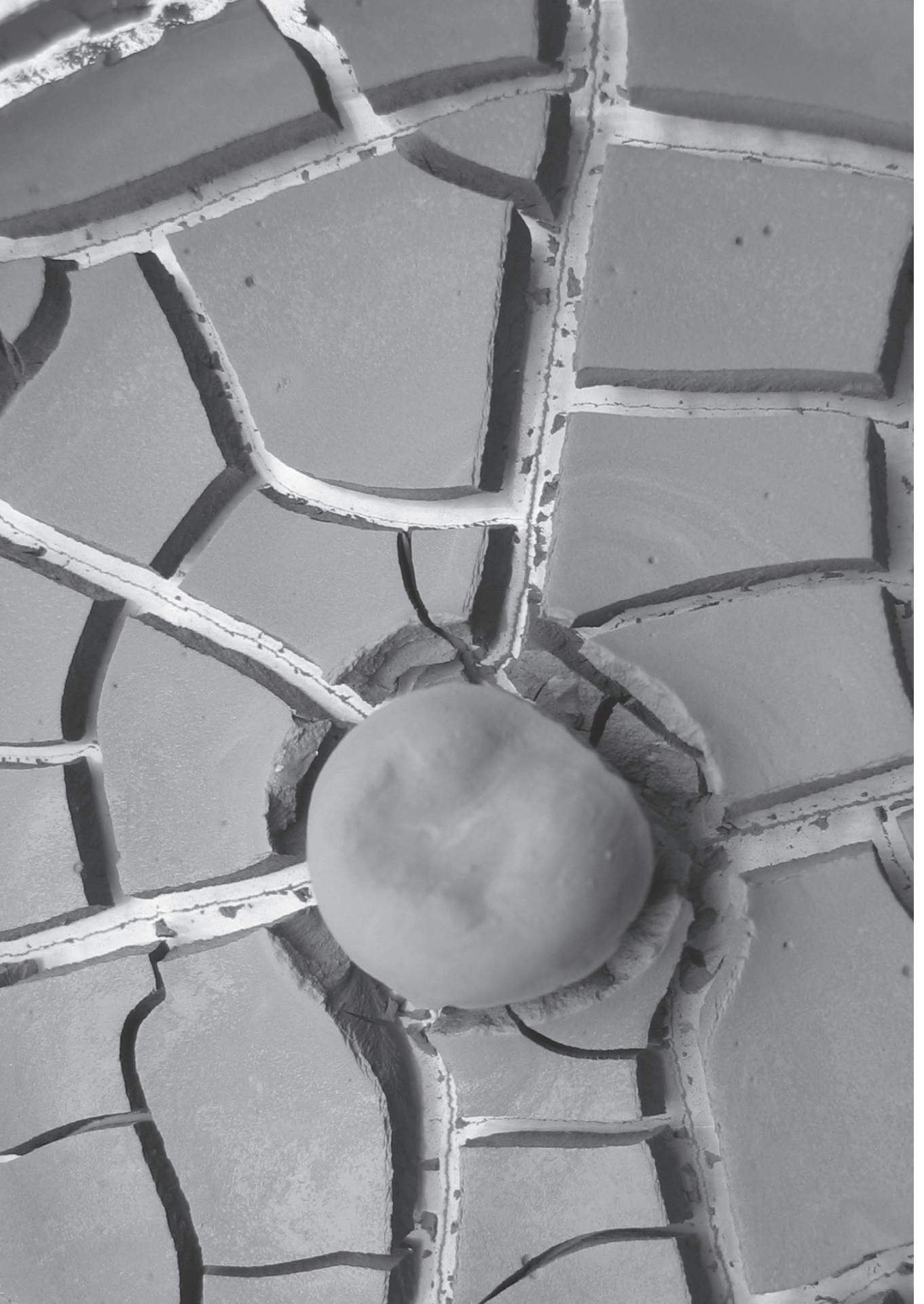

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Photo credits:

The picture of the industrial site on the cover was taken by photographer Hans Stakelbeek.

All other pictures in this thesis were taken during the various sampling campaigns and experimental work.